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INTERACTIONS OF SECOND MESSENGERS IN THE REGULATION OF GONADOTROPIN SECRETION FROM PITUITARIES OF DIESTROUS II AND OVARIECTOMIZED RATS

BY

©

SASWATI DAS

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of
the requirements for the degree of **DOCTOR OF PHILOSOPHY**

IN

ENDOCRINOLOGY

DEPARTMENT OF PHYSIOLOGY

EDMONTON, ALBERTA

FALL, 1991

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To my Father and Mother
for their
inspiration and encouragement

ABSTRACT

Ionomycin, phorbol 12-myristate 13-acetate (PMA) and flufenamate were used as probes to delineate the roles/interactions of the Ca^{++} and cAMP messenger systems as mediators of gonadotropin secretion from pituitaries of diestrous II and ovariectomized (OVX) (72 h) rats.

Quartered pituitaries from donor animals were perifused (0.25 mL/min) with Krebs Improved Ringer I bicarbonate medium (37°C), and sequential effluent fractions collected every 10 min. The samples were later assayed for LH and FSH by radioimmunoassay.

Infusions of GnRH to pituitaries from diestrous II rats resulted in a biphasic gonadotropin response: an initial, protein synthesis-independent component (phase I), followed 60 min later by a greatly augmented, protein synthesis-dependent component (phase II). While ionomycin evoked a small increase of gonadotropin secretion (phase I) (partially independent of protein synthesis), and PMA stimulated an equally small, delayed (70-80 min), protein synthesis-dependent component (phase II), the two agents interacted synergistically to generate a biphasic response.

Infusions of GnRH to pituitaries from OVX rats resulted in an attenuated, protein synthesis-dependent phase II response. The ionomycin-induced LH secretion was protein synthesis-independent, while FSH secretion was only partially dependent on protein synthesis. Although PMA alone did not stimulate gonadotropin secretion, it interacted synergistically with ionomycin to stimulate the secretion of the gonadotropins by protein synthesis-dependent mechanisms.

Flufenamate, which inhibited GnRH-stimulated cAMP production in pituitaries from OVX rats, selectively inhibited the GnRH-stimulated protein synthesis-dependent component of gonadotropin secretion. The flufenamate-inhibited secretion was reversed by dibutyryl cAMP, although the nucleotide alone was an ineffective secretagogue.

The results suggest that ionomycin and PMA can stimulate a biphasic gonadotropin secretion from pituitaries of diestrous II rats. Ca^{++} mobilization (ionomycin) appears to

mediate the initial secretion (phase I), PMA-activated mechanisms mediate the small, delayed, component of phase II secretion, and combined ionomycin- and PMA-stimulated mechanisms synergise to mediate the remaining augmented, phase II component of the biphasic response.

The attenuated secretion obtained from pituitaries of OVX rats includes an initial, small, cAMP-independent component mediated by Ca^{++} mobilization, and a larger component mediated by cAMP (indirectly), and synergistic interactions between ionomycin and PMA. Finally, the attenuation of the responses obtained from pituitaries of OVX rats appears to be due to the absence of the delayed, PMA-stimulated component of gonadotropin secretion.

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LIST OF ABBREVIATIONS AND SYMBOLS USED IN THE TEXT, FIGURES AND TABLE.

AA	: Arachidonic acid.
ACTH	: Adrenocorticotropic hormone.
ADP	: Adenosine 5'-diphosphate.
Arg	: Arginine.
ATP	: Adenosine 5'-triphosphate.
BSA	: Bovine serum albumin.
Ca^{++}	: Calcium ion.
CaM	: Calmodulin.
cAMP	: Cyclic adenosine 3', 5'-monophosphate.
cDNA	: Complementary deoxyribonucleic acid.
cf.	: Compare.
cGMP	: Cyclic guanosine 3', 5'-monophosphate.
cpm	: Counts per minute.
CT	: Cholera toxin.
DAG	: Diacylglycerol.
DBcAMP	: Dibutyryl cyclic adenosine monophosphate (N^6, 2'-O-dibutyryl cyclic adenosine 3', 5'- monophosphate).
DII	: Diestrous II stage of the female rat estrous cycle.
EDTA	: Ethylene diamine tetraacetic acid.
e.g.	: For example.
ER	: Endoplasmic reticulum.
FS	: Folliculo-stellate cells.
FSH	: Follicle-stimulating hormone.
Flu	: Flufenamic acid.

GDP : Guanosine 5'-diphosphate.

GH : Growth hormone.

G_i : Inhibitory G protein associated with the adenylyl cyclase enzyme.

Glu : Glutamic acid.

Gly : Glycine.

GnRH : Gonadotropin-releasing hormone.

G protein : Guanine nucleotide-binding protein.

Gpp(NH)p : 5'-guanylylimidodiphosphate.

G_s : Stimulatory G protein associated with the adenylyl cyclase enyme.

GTP : Guanosine 5'-triphosphate.

His : Histidine.

IBMX : Isobutyl methyl xanthine.

Iono : Ionomycin.

IPs : Inositol phosphates.

IP_3 : Inositol 1, 4, 5, trisphosphate.

IP_4 : Inositol 1, 3, 4, 5, tetrakisphosphate.

K^+ : Potassium ion.

kDa : Kilo Dalton — unit for expressing molecular mass.

KIRB : Krebs Improved.Ringer I Bicarbonate solution.

Leu : Leucine.

LH : Luteinizing hormone.

LTA_4 : Leukotriene A_4.

LTB_4 : Leukotriene B_4.

LTC_4 : Leukotriene C_4.

LTD_4 : Leukotriene D_4.

MPA : Mycophenolic acid.

mRNA : Messenger ribonucleic acid.

Na^+	: Sodium ion.
NaF	: Sodium fluoride.
NaI^{125}	: Sodium iodide (radioactive isotope of iodine).
NIDDK	: National Institute of Diabetes, Digestive and Kidney Diseases.
OVX	: Ovariectomized rat.
PBS	: Phosphate-buffered saline.
PDE	: Cyclic nucleotide phosphodiesterase enzyme.
PGE_2	: Prostaglandin E_2.
Pi	: Inorganic phosphate.
PIP_2	: Phosphatidyl inositol 4, 5, bisphosphate.
PKA	: cAMP-dependent protein kinase.
PKC	: Ca^{++}-activated, phospholipid-dependent protein kinase.
PMA	: Phorbol 12-myristate 13-acetate.
PPi	: Inorganic pyrophosphate.
PRL	: Prolactin.
Pro	: Proline.
PT	: Pertussis toxin.
RIA	: Radioimmunoassay.
Ser	: Serine.
SNP	: Sodium nitroprusside.
Trp	: Tryptophan.
TSH	: Thyroid-stimulating hormone.
(v/v)	: Volume/volume.
(w/v)	: Weight/volume.
5'-AMP	: Adenosine 5'-monophosphate.
5'-HPETE	: 5-hydroperoxy-6, 8, 11, 14-eicosatetraenoic acid.
8-bromo-cAMP	: 8-bromo cyclic adenosine 5'-monophosphate.

α : alpha.
β : beta.
δ : delta.
ε : epsilon.
γ : gamma.
π : pi
ζ : zeta.

Chapter I

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a neurohormone synthesized in cell bodies of peptidergic, neurosecretory cells located in the arcuate nucleus of the medial, basal hypothalamus in mammals (Okon and Koch, 1976 and Silverman et al., 1977). GnRH is transported from the cell bodies by axoplasmic flow in axons of the tuberoinfundibular tract to the axonal terminals in the median eminence area, where it is stored in secretory granules. These axonal terminals are closely juxtaposed to the primary capillary blood vessels of the hypothalamic-hypophyseal portal system which transports secreted GnRH to the anterior pituitary gland (Speroff et al., 1983).

The principal target cells of hypothalamic GnRH are the gonadotropes of the anterior pituitary (Catt et al., 1983). GnRH stimulates the release of the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the gonadotropes. The gonadotropins, in turn, regulate the gonads, mediating several physiological functions, one of them being the synthesis of steroid and peptide hormones. Thus, GnRH, gonadotropins and gonadal hormones are hormonally interconnected to form the hypothalamo-hypophyseal gonadal axis (Fink, 1979). The interplay among these hormones is controlled by different feedback processes.

The different feedback processes involved in the regulation of this axis include the long, short and the ultrashort feedback loops (Speroff et al., 1983). The long feedback loop involves the feedback effects of the gonadal hormones at the level of the hypothalamus and the pituitary. The effect can be either stimulatory (positive) or inhibitory (negative). The short feedback loop is a negative feedback effect of gonadotropins on GnRH secretion from the hypothalamus, thereby causing a decrease in pituitary secretion of gonadotropins. The ultrashort feedback system involves the inhibition by GnRH of its own synthesis (Speroff et al., 1983).

Besides these three control mechanisms, signals from higher centers in the central nervous system also modify GnRH secretion. The actions of the higher centers are predominantly mediated by two neurotransmitters, dopamine and norepinephrine. Norepinephrine probably exerts a stimulatory effect, while dopamine exerts an inhibitory effect, modifying the frequency of the pulsatile release of GnRH from the hypothalamus (Speroff et al., 1983).

Studies in a variety of mammals indicate that the secretion of GnRH is controlled by two hypothalamic centers in females: the tonic and the cyclic centers. The tonic center is located in the arcuate nucleus of the medial, basal hypothalamus (Kalra and Kalra, 1983 and Fink, 1988). This center controls the day to day secretion of GnRH, thereby regulating daily gonadotropin secretion during the reproductive cycle in females. It is responsive to the negative feedback effects of gonadal steroids. The cyclic center is located in the preoptic area of the anterior hypothalamus (Goodman, 1978, Kalra, 1983 and Fink, 1988). It is responsive to the positive feedback action of estradiol, causing a midcycle surge of GnRH and a subsequent preovulatory surge of gonadotropins (Goodman, 1978 and Speroff et al., 1983). In contrast, GnRH secretion in males is regulated by a tonic center located in the arcuate nucleus (Goodman, 1978, Kalra and Kalra, 1983 and Fink, 1988).

As stated earlier, hypothalamic GnRH is transported to the anterior pituitary where it stimulates the secretion of the gonadotropins. This action of GnRH is mediated by a number of intracellular compounds commonly referred to as "second messengers" or "intracellular mediators" of GnRH. The present study attempts to investigate the interactions of some of the intracellular mediators during the production of characteristic secretory profiles of gonadotropins in the presence or absence of ovarian factors.

Chapter II

LITERATURE REVIEW

A. The Anterior Pituitary

The primary target of hypothalamic GnRH is the gonadotropes of the pituitary gland. The pituitary gland or hypophysis is located below the hypothalamus in a small depression of the sphenoid bone, known as the sella turcica (Bloom and Fawcett, 1975). This endocrine organ is actually composed of three distinct components, the pars distalis (anterior lobe), the pars intermedia (intermediate lobe) and the pars nervosa (posterior lobe).

The anterior pituitary (pars distalis) represents the largest part of the hypophysis and originates as a dorsal outpocketing of the roof of the embryonic pharynx known as Rathke's pouch (Bloom and Fawcett, 1975). It is composed of secretory cells (including follicular cells) and non-secretory cells such as macrophages and fibroblasts. The secretory cells are arranged in irregular cords and clumps which remain intimately associated with an extensive system of thin-walled capillaries of the blood vascular system commonly referred to as sinusoids. Initially, the secretory cell types were classified on the basis of the staining properties of the granules. However, a more recent classification was developed based on the size and shape of secretory granules (Farquhar et al., 1975), as well as immunohistochemical staining procedures (Nakane, 1970).

It is now generally accepted that the anterior pituitary is a multiendocrine organ composed of at least five types of secretory cells. They are as follows: (1) the somatotropes which secrete growth hormone (GH); (2) the mammotropes or lactotropes which secrete prolactin (PRL); (3) the thyrotropes which secrete thyroid-stimulating hormone (TSH); (4) the corticotropes which secrete adrenocorticotropic hormone (ACTH) and related peptides and (5) the gonadotropes which secrete two hormones, the gonadotropins, FSH and LH. Immunocytochemical evidence in the rat suggests that each individual pituitary hormone is secreted from distinct cell types with the exception of LH and FSH. Recent studies using the centrifugal elutriation technique have revealed that there are two subtypes of gonadotropes. The larger gonadotropes store both gonadotropins and are referred to as multihormonal gonadotropes, while the smaller gonadotropes which store either FSH or LH are called monohormonal gonadotropes (Childs et al., 1983 and Lloyd and Childs, 1988).

In adult rats, gonadotropes constitute about 7% of the pituitary cells, while comprising about 13% of the cells in immature rat pituitaries (Catt et al., 1983). Although gonadotropes are scattered throughout the anterior pituitary gland, they tend to be concentrated in certain areas known as the "sex zones". These areas are the dorsocephalic portion of the pars distalis from which they extend along both sides of the pars intermedia (Baker, 1974). It is noteworthy that gonadotropes are found in close association with lactotropes (Nakane, 1970 and Sato, 1980). The lactotropes are polygonal cells found uniformly distributed throughout the rat anterior pituitary (Baker, 1974). They are relatively abundant in pituitaries obtained from cycling females, but occur less frequently in pituitaries of male rats (Farquhar et al., 1975 and Sato, 1980). Some of the lactotropes extend their cytoplasmic processes and envelop gonadotropes i.e. the lactotropes appear to assume a cup shape, surrounding the gonadotropes (Nakane, 1970 and Sato, 1980). Apparently, there is a physiological significance behind this close association of the gonadotropes and lactotropes. It has been suggested that gonadotropes can modify the secretory activity of the lactotropes through the release of a paracrine factor (Denef and Andries, 1983). Additionally, an inhibitory action of lactotropes on gonadotropin secretion has been suggested (Sato, 1980).

The presence of folliculo-stellate cells (FS) in the anterior pituitary deserves some discussion. These are stellate cells which have long cytoplasmic extensions and are clustered to form follicles (Allaerts and Denef, 1989). The long cytoplasmic processes interconnect with each other and were observed to extend between and around the secretory cells (Allaerts and Denef, 1989). Although the FS cells are not juxtaposed with any pituitary cell type in particular, they do establish contact through their long cytoplasmic extensions. Initially, these cells were thought of as contributors to the phagocytosis of damaged or dead cells (Farquhar et al., 1975), and/or degradation of excess storage products. However, folliculo-stellate cells were recently implicated in paracrine functions, ion-transport (resulting in the regulation of the ionic composition of interstitial fluid), as well as some immune activity (Allaerts and Denef, 1989 and Allaerts et al., 1990). With respect to the paracrine functions of the FS cells, it should be noted that these cells may regulate the activity of gonadotropes by secreting a hormone "follistatin" (Gospodarowicz and Lau, 1989), which presumably suppresses the release of FSH.

A brief description of the chemistry of the gonadotropins will be provided before discussing further details about the regulation of gonadotropin secretion.

B. Chemistry of LH and FSH

LH and FSH belong to the class of conjugated proteins known as glycoproteins. Glycoproteins are those proteins which contain carbohydrate groups attached covalently to the polypeptide chain (Vaitukaitis et al., 1976). The gonadotropins share common structural features. Both are heterodimeric proteins (Vaitukaitis et al., 1976 and Pierce and Parsons, 1981) consisting of 2 dissimilar subunits designated alpha (α) and beta (β). These subunits are held together by non-covalent forces (hydrogen bonds, hydrophobic interactions and electrostatic bonds) and are composed of different amino acid residues and carbohydrate moieties (Li, 1972). For example, LH has 2 polysaccharide moieties attached to the α subunit and 1 polysaccharide moiety attached to the β subunit (Li, 1972). The polysaccharides are attached to asparagine amino acid residues via glycosylamine linkages (Li, 1972). All of the carbohydrate sidechains terminate with sialic acid (Vaitukaitis et al., 1976). It should be noted that the α subunit is common to both of these hormones, while the β subunit is specific for each hormone and confers the immunological and biological specificity of LH and FSH (Vaitukaitis et al., 1976).

The dissociated α and β subunits are biologically inactive, but retain their immunoreactivity (Butt, 1969). Furthermore, enzymatic desialylation enhances the immunoreactivity of the molecules, while it decreases the biological activity (Vaitukaitis et al., 1976). Thus, there are significant differences between the sites responsible for the immunological and biological activities of the molecules. This is of paramount importance, since the measurement of gonadotropins is routinely accomplished by radioimmunoassay techniques. As a result, the estimates obtained by these measurements may not be representative of biologically active hormones.

C. Synthesis of LH and FSH

The following discussion on the synthesis and secretion of gonadotropins is based on the work by Farquhar et al., (1975), as well as the classical scheme proposed by Palade (1975), for exocrine pancreatic cells. This secretory process was found to be applicable to most protein - secreting cells which possess storage granules e.g. exocrine cells, endocrine cells and leukocytes. However, the parenchymal cells of the liver, plasma cells and fibroblasts are exceptions to the following scheme, since they do not possess typical storage granules.

Synthesis of the hormones begins on the membranes of the rough endoplasmic reticulum (ER), by aggregated ribosomes (polysomes) which translate the genetic code in the form of messenger RNA (mRNA) into a specific sequence of amino acids (Jamieson, 1975). Recent evidence indicates that the α and β subunits of the gonadotropins are coded for by separate genes (Wilson et al., 1990). Once synthesized by the ribosomes attached to the ER, the polypeptide chains are transferred vectorially into the cisternae of the ER, presumably via specific pores in the membrane (Sabatini and Blobel, 1970 and Blobel and Dobberstein, 1975).

As well as functioning as a convenient conduit for onward shipment of the proteins, the ER also serves as the site for their chemical processing. The ER contains enzymes embedded in or attached to the inner surface of its membrane. These enzymes catalyse the production of disulphide bonds and attachment of the proximal sugars at appropriate points on the protein chains destined to become glycoproteins (Jamieson, 1975). In fact, both α and β subunits of LH and FSH are glycosylated at specific asparagine residues and are cross-linked internally by disulphide bonds (Pierce and Parsons, 1981). It is probably at this point that carbohydrates are linked to asparagine residues in the nascent peptide chain (Farquhar et al., 1975). It should be noted that GnRH enhances glycosylation and sulphation of both LH and FSH (Menon et al., 1977 and Wilson et al., 1990).

Following synthesis in the rough ER, the secretory proteins move into spaces referred to as transitional elements. These are portions of the ER devoid of polysomes (Jamieson, 1975). Evidence obtained from combined autoradiographic and cell fractionation experiments in the pancreatic cell suggests that these transitional elements bud off the ER as small transporting vesicles and carry their load of secretory proteins towards the Golgi complex. These transporting vesicles probably fuse with the membranes of the Golgi complex delivering their contents to the organelle (Jamieson, 1972).

The Golgi cisternae, consisting of a series of flattened, smooth-surfaced saccules stacked one on top of the other, as well as some associated vesicles and vacuoles, serve as a receiving and packaging site in the cell (Bloom and Fawcett, 1975). The secretory proteins undergo extensive chemical modifications inside the Golgi cisternae which contain the enzymes - glycosyl transferases in the inner surface of the Golgi membrane. These enzymes mediate the attachment of the polysaccharide moieties of the glycoproteins to the polypeptide chains (Jamieson, 1975). In most secretory cells, the saccules closest to the concave side are gradually filled with secretory material, become spherical and eventually bud off as immature storage granules to move towards the center of the cup-shaped area of the Golgi. The passage of the secretory proteins through the various cisternae of the Golgi complex occurs by simple diffusion (Jamieson, 1975). The immature storage granules are surrounded by a smooth membrane and contain proteins at a concentration less than that of mature granules. Gradually, with maturity, the protein concentration in the granules increases and the vacuoles are referred to as "condensing vacuoles" (Jamieson, 1972). Once the mature secretory granules are formed, the hormone remains in a dense insoluble form until its discharge from the cell or into lysosomes.

D. Secretion of LH and FSH

On the basis of morphological evidence obtained from electron microscopy, it is now generally accepted that secretion of anterior pituitary hormones occurs by "exocytosis" (Farquhar et al., 1975 and Farquhar, 1977). During exocytosis, the membranes of the granules apparently fuse selectively with the portion of the plasma membrane that faces the capillary basement membrane, resulting in the ejection of the granular contents into the perivascular tissue spaces (Farquhar et al., 1975). The hormones are then rapidly solubilized and diffuse through the remaining structural barriers (parenchymal basement membrane, endothelial basement membrane and fenestrated areas of the capillary endothelial cells) to enter the circulation.

It should be mentioned that the above scheme of exocytosis was outlined as a general proposal. Although the proteins considered in the above discussion are packaged into secretory granules, there is no definite information as to whether all exportable proteins in secretory cells must follow the above pathway to be packaged prior to release.

An involvement of the microtubule-microfilament system in exocytosis has been proposed by a number of investigators (Farquhar et al., 1975, Farquhar, 1977 and Ravindra and Grosvenor, 1990). Functional evidence for an involvement of the cytoskeleton elements in the secretion of hormones from the anterior pituitary arises from the fact that drugs such as colchicine, vincristine and vinblastine, which bind to microtubule proteins (polymers of tubulin) and cytochalasin B which affects actin polymerization (formation of microfilaments), inhibit anterior pituitary hormone secretions (Farquhar et al., 1975).

Vinblastine and colchicine did not affect GnRH-stimulated LH and FSH secretion from rat hemipituitaries in vitro (Pickering and Fink, 1979), dispersed rat anterior pituitary cells (Conn and Hazum, 1981), or dispersed anterior pituitary cells of ovariectomized sheep (Adams and Nett, 1979[a]). The two drugs did depress the basal release of LH from dispersed ovine cells (Adams and Nett, 1979[a]). In contrast to the anti-microtubule drugs, the anti-microfilament drugs (cytochalasins), were found to inhibit gonadotropin secretion from rat hemipituitaries in response to GnRH in vitro (Pickering and Fink, 1979). Interestingly, cytochalasin B stimulated basal LH secretion, while reducing GnRH-stimulated LH release from dispersed ovine pituitary cells (Adams and Nett, 1979[a]). The results suggest that microtubules probably facilitate the movement of packaged secretory granules from the Golgi apparatus to the plasma membrane by acting as tracks along which the granules can move (Ravindra and Grosvenor, 1990). In contrast, the microfilaments are probably involved in the secretory process activated by GnRH (Ravindra and Grosvenor, 1990). Additionally, the binding of actin to anterior pituitary secretory granules also suggested a role for actin in the process of exocytosis (Ravindra and Grosvenor, 1990).

Recently, another heterogenous group of proteins, known as intermediate filaments, were identified as components of the cell cytoskeletal system (Ravindra and Grosvenor, 1990). The intermediate filaments apparently interact with the microtubules and microfilaments, holding the other cytoskeletal elements together (Ravindra and Grosvenor, 1990). The role(s) of the intermediate filaments in hormone secretion are unknown due to the unavailability of drugs that specifically interact with the intermediate elements (Ravindra and Grosvenor, 1990). In this regard, it should be noted that the specificity of the other cytoskeletal drugs is questionable, because they also bind to plasma membranes, inhibit transport mechanisms and disrupt cell energetics (Farquhar et al., 1975 and Ravindra and Grosvenor, 1990). As a result, the secretory functions attributed to the cytoskeletal system may actually be due to other effects of the drugs.

E. Crinophagy

Secretory granules in pituitary cells can also fuse with lysosomes, to be degraded by a process called "crinophagy". This phenomenon was first described in mammotropes (Farquhar et al., 1975 and Farquhar, 1977), and represents a mechanism for removing excess hormone. In addition to mammotropes, the process also occurs in gonadotropes (Rommler et al., 1978), where increased lysosomal activity was observed after in vivo bolus infusions of supraphysiological amounts of GnRH. This increased activity was correlated with a decrease in pituitary LH content to levels which existed prior to the administration of the hypothalamic hormone, i.e. the decrease in LH was not due to secretion of the hormone.

Pituitary lysosomes are known to contain proteases capable of successively degrading pituitary hormones into inactive oligopeptides and dipeptides (Farquhar, 1977). The latter are capable of diffusing through the lysosomal membrane into the cytosol, where they are further degraded into their constituent amino acids and restored to the metabolic pool for reutilization by the pituitary cells (Farquhar, 1977).

F. Secretory Profiles of LH and FSH

The endogenous in vivo secretion of LH and FSH from anterior pituitaries is now known to occur in a pulsatile manner, in response to pulsatile discharges of GnRH (Speroff et al., 1983). The availability of synthetic GnRH has facilitated studies on the releasing action of the decapeptide. Indeed, in vivo studies in rats (Gay et al., 1969 and Ratner et al., 1976) have demonstrated a 4 to 5-fold peak increase in plasma LH levels within 10 to 15 minutes of a single intravenous injection of GnRH, with the secretion quickly returning to basal levels. However, sequential pulsatile administration of equal amounts of GnRH *in vivo* in women (Wang *et al*., 1976) and in cycling, female rats (Aiyer *et al*., 1974), as well as *in vitro* using pituitary tissue blocks from intact, cycling female rats (Edwardson and Gilbert, 1976, Bourne and Baldwin, 1980 and Waring and Turgeon, 1980) resulted in an initial response to the first pulse, followed by an augmented response to the second pulse of GnRH. This process, by which a second challenge by the same concentration of GnRH generates a greater LH response than the first challenge is known as the "self-priming" effect of GnRH.

The priming effect is only observed in intact female rats with the greatest response occurring at proestrus (Aiyer *et al*., 1974). This phenomenon was attributed to estradiol, since ovariectomized (OVX) female rats did not demonstrate the priming effect (Aiyer *et al*., 1976 and Waring and Turgeon, 1980), while estradiol replacement resulted in the restoration of the phenomenon (Aiyer *et al*., 1976).

Additional studies also indicate that the expression of self-priming requires a minimum of 45-60 min to develop (Pickering and Fink, 1976), and is dependent on *de novo* protein synthesis (Pickering and Fink, 1976). Cycloheximide, an inhibitor of translation (Lehninger, 1975) reduced the second response to GnRH to the level of the first response without appreciably affecting the first response (Edwardson and Gilbert, 1975 and Pickering and Fink, 1976).

Continuous infusions of GnRH *in vivo* yielded a biphasic response in women (Wang *et al*., 1976), and female rats (Blake, 1976). Biphasic secretions were also observed *in vitro* using anterior pituitary tissue blocks from intact, cycling female rats (Dekoning *et al*., 1976, Bourne and Baldwin, 1980 and Baldwin *et al*., 1983). The biphasic response is characterized by an immediate, low secretion of the gonadotropins (termed phase I). Approximately 50-90 minutes later, an additional, augmented component of gonadotropin secretion became apparent (termed phase II) (DeKoning *et al*., 1976, Bourne and Baldwin, 1980 and Bourne and Baldwin, 1987[a]). Studies also indicated that the phase II component of the biphasic secretion is protein synthesis-dependent, since this phase of secretion was selectively abolished by cycloheximide (Dekoning et al, 1976, Bourne and Baldwin, 1980 and Baldwin et al., 1983).

Interestingly, the secretory profiles of gonadotropins from pituitaries of OVX rats are different from those obtained from pituitaries of cycling female rats. GnRH-stimulated LH secretion from pituitaries of OVX rats was characterised by a secretory profile in which the augmented secretion of the phase II component was attenuated (Baldwin et al., 1983).

The secretory profiles of LH and FSH obtained from pituitaries of male rats are different from those obtained from female pituitaries (Bourne and Baldwin, 1987[a,b]). Continuous infusions of GnRH for 4 hours to pituitaries from males resulted in a slow, steady increase in LH secretion for 2.5 hours, at which time the secretory rate stabilized until GnRH infusion was terminated. Similar profiles in FSH secretion were also observed, with the difference that FSH release was rather prompt (Bourne and Baldwin, 1987[b]). Unlike intact female rats, the gonadotropin responses to continuous infusions of GnRH from pituitaries of male rats were completely inhibited by cycloheximide (Bourne and Baldwin, 1987[b]).

Interestingly, in contrast to pituitaries in intact animals and their derived pituitary tissue blocks, sequential bolus infusions of equal amounts of GnRH to dispersed female anterior pituitary cells in vitro did not elicit a self-priming effect (Naor et al., 1982 and Chang et al., 1987). Furthermore, continuous infusions of GnRH to dispersed male pituitary cells yielded a biphasic response which was characterized by a rapid, initial rise in LH secretion peaking within 1-2 min of GnRH infusion and lasting for 5 min. This was termed the first peak and was followed by a more gradual and sustained phase of release over the next 90 min, termed the second peak (Hopkins, 1977). Biphasic responses from dispersed, female anterior pituitary cells were characterized by a rapid initial phase with a peak within 1 min of GnRH infusion, which lasted for 3-4 min. The rapid response was followed about 5-6 min later by a lower rate of LH release (Naor et al., 1982 and Catt et al., 1983). It should be emphasized that the biphasic responses obtained from dispersed cells have different temporal characteristics compared to the responses obtained in vivo or in vitro using pituitary tissue blocks.

The fact that the in vitro responses obtained from dispersed pituitary cells do not duplicate in vivo secretory profiles and phenomena, while pituitary tissue blocks do, affords some degree of confidence that the responses obtained from anterior pituitary tissue blocks might be duplicating in vivo responses.

G. Structure of GnRH

GnRH is a decapeptide with a linear amino acid sequence as follows: Pyro-Glu^1-His^2-Trp^3-Ser^4-Tyr^5-Gly^6-Leu^7-Arg^8-Pro^9- Gly^{10}-NH$_2$. The conformation of the molecule is stabilized by the formation of a hydrogen bond between the pyrrolidone carbonyl of residue 1 and the glycinamide group of residue 10 (Nikolics et al.,1977 and Coy et al.,1979), such that the two termini are in close proximity and the molecule remains in the lowest energy conformation (Momany,1976).

The significance of this conformation lies in the fact that the contiguity of pyro-Glu and Gly-amide is believed to form a region that is important for binding of the decapeptide to specific GnRH receptors (Coy et al., 1979). Substitutions in either or both of these positions result in a decreased binding affinity for the receptor and consequently, a reduction in GnRH-receptor binding (Coy et al., 1979). It also appears that the first three amino acids are involved in effector activation i.e., activation of the second messenger systems (Conn et al., 1987[a]). D-amino acid substitutions in the first three amino acid positions result in the production of pure antagonists i.e., the molecules will bind to the receptor, but will not activate the effector systems (Conn et al., 1987[a]).

The GnRH molecule is also sensitive to biological degradation. The preferred site of proteolytic attack is adjacent to the Gly^6 position (Conn et al., 1987[a]). Replacement of this sixth residue with D-amino acids containing bulky, hydrophobic substituents yields metabolically stable compounds. The presence of D-amino acids at this position also stabilizes a beta turn in the molecule needed for its biologically active conformation (Coy et al.,1976 and Conn et al.,1987[a]). In addition to the replacement of Gly^{6} with D-amino acids, substitution of the Gly^{10} with an ethylamide group yields molecules which have higher receptor affinities and effector potencies than GnRH (Fujino et al., 1974). Consequently, these substitutions have resulted in the production of various superagonists of GnRH.

H. Mechanism of GnRH Action

It is commonly accepted that the principle actions of GnRH are: (1) stimulation of gonadotropin secretion, and (2) stimulation of de novo protein synthesis. This includes the biosynthesis of LH and FSH, as well as other proteins involved in gonadotrope cell function (McCann, 1971 and Labrie et al., 1973).

Although a number of postulated intracellular mediators are implicated in the actions of GnRH, the specific mechanisms involved are still unknown. The ensuing discussion is a review of the present knowledge of the mechanism of action of GnRH.

I. GnRH-Receptor Interaction

The initial step in the mechanism of action of GnRH is the reversible, binding of GnRH to specific, high affinity, low capacity receptors ($Ka = 10^{8} - 10^{9}\ M^{-1}$) located on the plasma membranes of gonadotropes (Clayton et al., 1979[a], Marshall et al., 1980 and Clayton and Catt, 1981). The formation of GnRH-receptor complexes is due to non-covalent bonds. The non-covalent bonds involve electrostatic interactions including hydrogen bonding, hydrophobic interactions and Van der Waals forces (Baxter and Funder, 1979). It is generally accepted that electrostatic interactions take place between oppositely charged groups on the hormone and its complementary site on the receptor. These forces plus hydrogen bonding are believed to play a role in the initial recognition of the hormone by the receptor (Baxter and Funder, 1979). Van der Waals forces are due to electrical attractions between oppositely charged transient dipoles which require an exact intra-atomic distance to develop. These forces are generally believed to play an initial, but crucial role in the establishment of hydrophobic interactions (Baxter and Funder, 1979). Finally, the hydrophobic interactions serve as the driving force for binding, since hydrophobic regions of the hormone seek contact with similar hydrophobic regions on the receptor (Baxter and Funder, 1979).

An alternate model has been postulated for GnRH-receptor complex formation (Hazum and Conn, 1988). The belief is that the driving force for the complex formation is probably an ionic interaction between the positively charged amino acid arginine at position 8 and the negatively charged carboxyl groups in the binding site of the receptors (Hazum, 1987). Furthermore, the hormone-receptor complex is believed to be stabilized by aromatic π-π interactions between the histidine, tryptophan and tyrosine residues of the hormone, and the tyrosine and tryptophan residues in the binding site (Keinan and Hazum, 1985 and Hazum and Conn, 1988).

In unstimulated gonadotropes, the unoccupied GnRH receptors are uniformly distributed in the plasma membrane. Following the binding of GnRH to receptors, the receptors tend to cross-link or dimerize with each other, a process referred to as "microaggregation" (Conn et al., 1987[a]). This process is followed by clustering or patching of the receptors and subsequent internalization of hormone-receptor complexes into the cell via coated pits and vesicles (Hazum and Conn, 1988). The receptor complexes are then transferred to endosomes. From this step onward, the hormone-receptor complexes might have two different fates. Some of the complexes are transported to lysosomes and degraded, while the others are carried to the Golgi cisternae where the receptors are recycled back to the plasma membrane via secretory granules (Hazum and Conn, 1988).

Several studies indicate that the process of patching and the internalization of receptors are not involved in mediating the secretion of gonadotropins. When these processes were prevented by immobilizing GnRH through covalent attachment to agarose beads, the decapeptide was still effective in stimulating gonadotropin secretion (Conn et al., 1981[c]). Additionally, vinblastine, a drug that blocks the formation of patches (i.e. large receptor aggregates), and internalization of the GnRH receptor, did not block GnRH-stimulated LH release (Conn and Hazum, 1981). Furthermore, after internalization of GnRH receptors had occurred and all extracellular GnRH was removed, the secretion of LH was promptly terminated (Conn et al., 1987[a]). Taken together, these experiments suggest that patching and internalization are not required for initiating gonadotropin release, nor are they required to support the ongoing release process (Conn et al., 1987[a]).

In contrast to the situation obtained with patching and internalization, a role for microaggregation in signal transduction of LH secretion was demonstrated. In an elegant study, gonadotrope activation was shown to be dependent on GnRH receptor dimerization (microaggregation) brought about by the movement of the receptors within a critical distance of less than 15 nm and greater than 1.5 nm (Conn et al., 1982, Conn et al., 1987[a] and Hazum and Conn, 1988). Since GnRH is neither sufficiently long nor multivalent, Conn et al. (1982), postulated that microaggregation is initiated by GnRH occupancy of the receptor which may expose complementary binding sites on the receptors, facilitating dimerization. Another suggestion was that occupancy of receptors may activate enzymes which catalyze disulphide or peptide bond formation, thereby cross-linking the receptors (Conn et al., 1982).

Essentially, the initial step of GnRH-receptor complex formation is followed by microaggregation, which appears to play a pivotal role in signal transduction. The activation of the signal transduction mechanisms results in an increase in the intracellular concentrations of a number of chemical substances known as "second messengers" or "intracellular mediators" of GnRH. Coupling of receptor occupancy to the generation of the intracellular mediators is brought about by guanine-nucleotide binding proteins or "G proteins" (Conn et al., 1987[a] and Gilman 1987).

II. G Proteins

Guanine nucleotide-binding proteins or G proteins are a class of heterotrimeric, membrane-associated proteins (Levitzki 1986), composed of alpha (α), beta (β), and gamma (γ) subunits named in order of decreasing mass (Gilman, 1987 and Brown and Birnbaumer, 1988). The α subunit is unique for each type of G protein and, to date, seven distinct cDNAs have been cloned for this subunit (Brown and Birnbaumer, 1988). In contrast to α subunits, β and γ subunits are highly homologous and functionally interchangeable. This allows them to interact with the different α subunits of a cell. The β and γ subunits exist as two types of complexes: the β36γ and β35γ, with the numbers referring to the mass of the β subunit in kDa (Gilman,1987).

The α subunit contains the guanine nucleotide-binding site. In the nonactivated state the site is occupied by GDP which is exchanged for GTP during activation. The α subunit also has intrinsic GTPase activity that permits the slow hydrolysis of GTP to GDP (Levitzki,1986, Gilman,1987 and Weiss et al.,1988).

The following generalized scheme of G protein activation is based on modifications of the "collision-coupling" and "dissociation" models (Levitzki, 1986). The important feature of this model is the fact that G and the catalytic or effector component (C) always remain tightly bound to form a GC complex (Levitzki, 1986). The first step in G protein activation is the binding of the hormone to the receptor to form a HR complex. The interaction of the HR complex with the GC complex (G is always bound to C [γβα C]), requires the presence of βγ subunits because in their absence GDP/GTP exchange cannot occur (Levitzki, 1986). In other words, the stimulatory receptor interacts with the intact GC (γβα C) to bring about the GDP to GTP exchange. The γβα(GTP).C then dissociates (Northup et al., 1983 and Katada et al., 1984) to the activated form α'(GTP).C' transiently, and readily recombines with the $\gamma\beta$ subunits as soon as GTP is hydrolyzed to GDP (Levitzki, 1986). The model is outlined in the following scheme:

(1) $H + R <=> HR$

(2) $HR + GC\ (\gamma\beta\alpha_{.GDP}\ C) + GTP <=> HRG'C'\ (\gamma\beta\alpha'_{.GTP}\ C') + GDP$

(less active species)

(3) $HRG'\ C'\ (\gamma\beta\alpha'_{.GTP}C') <=> HR + \alpha'_{.GTP}C' + \gamma\beta$

(active species)

(4) $\alpha'_{.GTP}C' + \gamma\beta \xrightarrow[GTPase]{} G\ C\ (\gamma\beta\alpha_{.GDP}C) + P_i$

where: H represents hormone

R represents receptor

G represents G protein

C represents catalytic component

P_i represents inorganic phosphate

' denotes activated form)

The effector systems that are known to be coupled to G proteins are the hormone - sensitive adenylyl cyclase (it interacts with both stimulatory [G_s] and inhibitory [G_i] G proteins), light-regulated retinal rod cyclic GMP phosphodiesterase (G_t), hormone regulated phospholipase C (G_p) (Levitzki,1986 and Gilman,1987) and phospholipase A_2 (Fain et al., 1988). Some G proteins act directly on K^+ channels in human red blood cells and neurosecretory cells. These channels are known as G protein-gated K^+ channels (Brown and Birnbaumer, 1988). The G_s protein which stimulates adenylyl cyclase activation was also found to gate Ca^{++} channels in cardiac and skeletal muscle T-tubules (Mattera et al., 1989). Contrary to previous belief, this represents an example where one G protein affects two distinct effector systems (Mattera et al., 1989). It should be noted that binding of GTP to the G protein also facilitates the dissociation of hormones from the G protein-linked receptor (Gilman, 1987). This is an example of heterotropic negative cooperativity.

G proteins are involved in signal transduction mechanisms of GnRH. Perrin et al. (1989) demonstrated that guanylyl nucleotides inhibit the interaction of GnRH with its receptor in a manner characteristic of receptors that are coupled to G proteins. The use of 5'-guanylylimidodiphosphate [Gpp(NH)p], a nonhydrolyzable GTP analog inhibited GnRH agonist, but not antagonist, binding to its receptor. GTP and GDP also inhibited binding to a lesser extent, whereas other nucleotides had no effect. Perrin et al. (1989), concluded that the GnRH receptor appears to be coupled to GTP-binding proteins.

Andrews et al. (1986) also indicated that a GTP-binding protein is probably the coupling factor for GnRH receptors and phospholipase C. The active enzyme stimulates the accumulation of inositol phosphates. Andrews et al. (1986), demonstrated that GTP and other guanylyl nucleotide analogs (guanylylimidodiphosphate [GMP-PNP]) stimulated a time - and dose-dependent release of LH as well as inositol phosphate accumulation.

In short, it appears that the formation of GnRH receptor complexes and microaggregation result in the activation of G protein(s) which ultimately mediate the generation of some, if not all, of the second messengers of GnRH. To date, compounds which have been implicated as mediators of GnRH action are: calcium (Ca^{++}), diacylglycerol (DAG), inositol 1,4,5,trisphosphate (IP_3) (as well as other inositol phosphates [IPs]), cyclic adenosine 3', 5'-monophosphate (cAMP), cyclic guanosine 3', 5'-monophosphate (cGMP) and arachidonic acid (AA) and its metabolites (Catt et al., 1983 and Conn et al., 1987[a]). The roles of these second messengers will be described in the following sections.

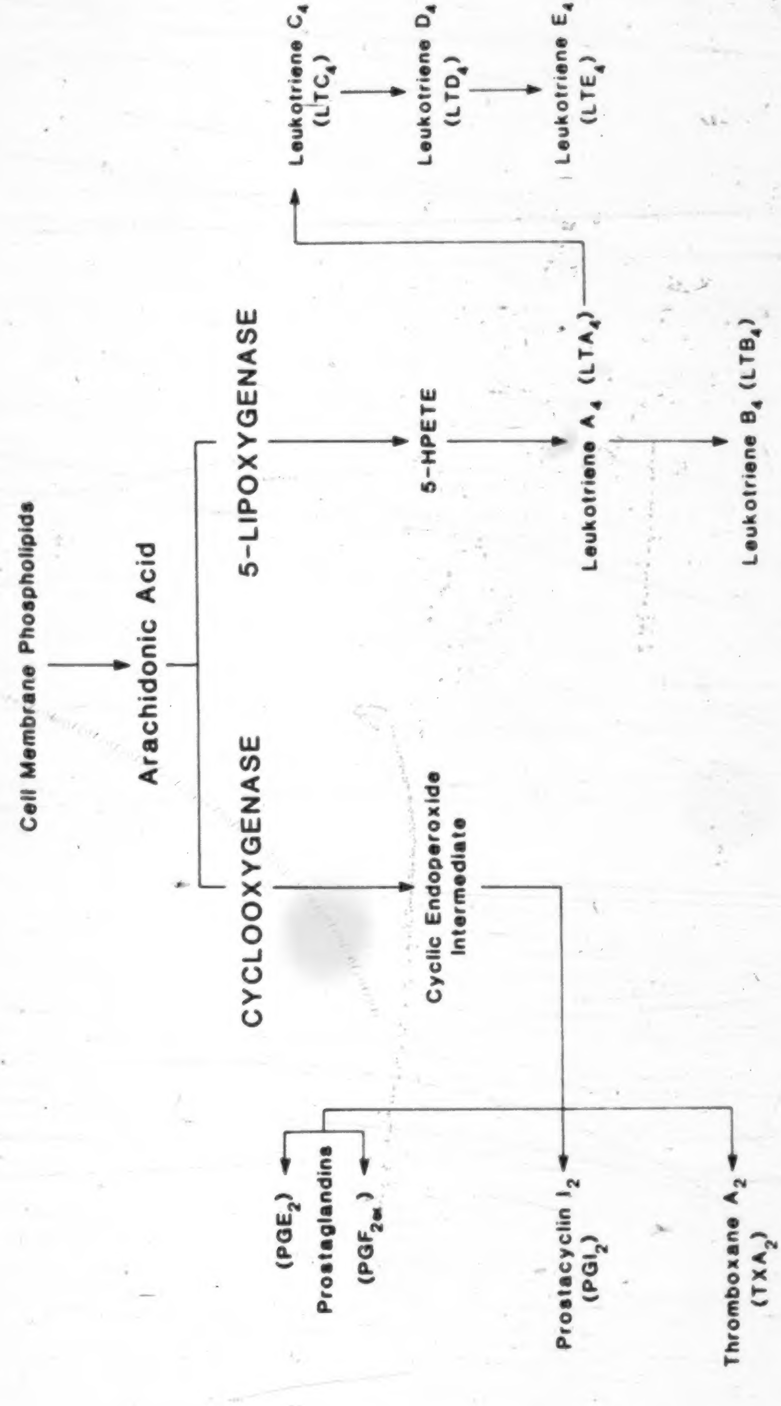
III. Role of Arachidonic Acid and its Metabolites

In a wide variety of mammalian tissues, agonist-stimulated hydrolysis of membrane phosphoinositides results in the generation of AA and subsequent production of its metabolites (Abdel-Latif, 1986). In most mammalian plasma membrane phosphoinositides, the fatty acid at the C_1 position is usually stearate while AA is exclusively found at the C_2 position. The AA can be released from the phosphoinositides by the action of specific phospholipases (Irvine, 1982). Two pathways have been proposed for the release of AA. The hydrolysis of phosphoinositides can proceed via an indirect pathway stimulated by phospholipase C, which produces DAG. The DAG is subsequently hydrolyzed by DAG- and monoacyl glycerol - lipases to release AA (Irvine, 1982 and Abdel-Latif, 1986). The second pathway involves the direct release of AA from phospholipids by phospholipase A_2 (Irvine, 1982 and Abdel-Latif, 1986). Apparently, both phospholipase C and phospholipase A_2 pathways are involved in AA liberation from phosphoinositides. However, neither the relative contribution of each of these pathways to AA generation, nor the relationship between the two pathways is known.

Once generated from membrane phospholipids, AA is converted into a group of compounds known as eicosanoid derivatives (Abdel-Latif, 1986). The eicosanoid derivatives include prostaglandins, prostacyclins, thromboxanes, and leukotrienes. It should be noted that the limiting factor in the production of eicosanoids is the availability of AA. One of the major pathways of AA metabolism is its conversion by the cyclooxygenase enzyme to a cyclic endoperoxide intermediate. This intermediate can be converted into prostaglandins, prostacyclins and thromboxanes (Fig.1). The second major pathway involves the enzyme 5-lipoxygenase, which converts AA to 5-hydroperoxy-6, 8, 11, 14-eicosatetraenoic acid (5-HPETE). The 5-HPETE can be converted into leukotriene A_4 (LTA_4). This compound in turn can subsequently be metabolized to leukotriene B_4 (LTB_4) or leukotriene C_4 (LTC_4) which is a precursor for leukotriene D_4 (LTD_4) and leukotriene E_4 (LTE_4) (Fig.1).

The recognition of AA as a potential mediator of GnRH actions was initially established by the fact that GnRH caused a 35% increase in arachidonic acid release from prelabeled phospholipids in cultured pituitary cells (Naor and Catt, 1981). This effect of

FIG. 1. Schematic outline of the metabolism of arachidonic acid into its cyclooxygenase and lipoxygenase products.


Cell Membrane Phospholipids
Arachidonic Acid
CYCLOOXYGENASE
5-LIPOXYGENASE
Cyclic Endoperoxide Intermediate
5-HPETE
(PGE_2)
Prostaglandins
$(PGF_{2\alpha})$
Prostacyclin I_2 (PGI_2)
Thromboxane A_2 (TXA_2)
Leukotriene A_4 (LTA_4)
Leukotriene B_4 (LTB_4)
Leukotriene C_4 (LTC_4)
Leukotriene D_4 (LTD_4)
Leukotriene E_4 (LTE_4)

GnRH on AA generation was found to be Ca^{++}-dependent and apparently did not result in prostaglandin formation (Naor and Catt, 1981). Addition of exogenous AA caused a dose-related release of LH, a response which occurred in the absence of extracellular Ca^{++} (Naor and Catt, 1981). It was therefore concluded that Ca^{++} is required at a step prior to arachidonic acid formation, possibly at phospholipase A_2-catalyzed hydrolysis of membrane phospholipids. Phospholipase A_2 is a membrane-bound enzyme (Kiesel et al., 1987[a]) that is activated by Ca^{++}-calmodulin complexes (Conn et al., 1981[a]).

Additional experiments demonstrated a dose-dependent stimulation of LH secretion in response to exogenously administered phospholipase A_2 and melittin (an activator of phospholipase A_2). The stimulatory effects of melittin and phospholipase A_2 on gonadotropin release were blocked by chloroquine and quinacrine, inhibitors of phospholipase A_2 (Naor and Catt, 1981 and Kiesel et al., 1987[a]).

The fact that AA is rapidly metabolized inside the cell suggested that its effects as a mediator of GnRH actions might be due to one or more of its different metabolites. Since prostaglandin concentrations were not increased during GnRH action, and since PGE_2 did not stimulate LH release (Naor and Catt, 1981), a role for the cyclooxygenase metabolites was excluded, and a role for the metabolites of the lipoxygenase pathway was actively investigated.

A role for the metabolites of the lipoxygenase pathway was demonstrated through the use of inhibitors of the lipoxygenase enzyme. Nordihydroguaiaretic acid (NDGA), an inhibitor of 5-lipoxygenase, inhibited the GnRH-induced LH secretion in a dose-dependent manner (Catt et al., 1983). It also inhibited the AA-stimulated secretion of LH (Conte et al., 1986), as well as the stimulatory actions of exogenously administered 5-lipoxygenase (Przylipiak et al., 1990). Interestingly, indomethacin, a cycloxygenase inhibitor, potentiated the actions of GnRH, probably by increasing the availability of AA and its lipoxygenase metabolites (Catt et al., 1983 and Conte et al., 1986).

Direct evidence for implicating these metabolites was established by the demonstration that 5-HPETE and 15-HPETE were more potent than AA in stimulating LH release from rat pituitary cells (Kiesel et al., 1986[a]). The effects of the HPETE compounds appear to be due to their conversion to leukotrienes since LTA_4, LTB_4, LTC_4 and LTE_4 all stimulated LH release (LTD_4 was however ineffective) (Kiesel et al., 1987[b]). Among the leukotrienes, LTC_4 was very potent in stimulating LH release and apparently duplicated the GnRH-stimulated biphasic secretion of LH obtained from dispersed pituitary cells (Kiesel et al., 1986[a]).

It should be readily apparent that there is ample evidence for a role of AA and its lipoxygenase metabolites in the mechanism of GnRH action on the pituitary gonadotropes. Current evidence indicates that AA and its metabolites are probably involved in the rapid, initial phase of the biphasic secretion of LH induced by GnRH from dispersed pituitary cells (Chang et al., 1987).

IV. Role of Cyclic GMP

The demonstration that GnRH increased cGMP levels in hemipituitaries of both male and female rats (Naor et al., 1978[a]), in dispersed pituitary cells and in enriched gonadotrope fractions (Naor et al., 1980 and Snyder et al., 1980) suggested that the nucleotide may play a role in GnRH actions. This notion was strengthened by the demonstration of a positive correlation between the concentration of cGMP produced and receptor occupancy by GnRH (Naor and Catt, 1980), and the fact that the GnRH-induced increase in cGMP production preceded LH release (Nakano et al., 1978). Interestingly, cGMP formation was dependent on extracellular calcium (Naor et al., 1980 and Catt, et al., 1983).

However, in other studies, blockade of GnRH-induced cGMP production by mycophenolic acid (MPA) did not impair GnRH-stimulated secretion. Furthermore, elevation of cGMP levels by sodium nitroprusside (SNP), or administration of cGMP or its analogs, did not stimulate LH release (Naor and Catt, 1980), although Nakano et al., (1978) did indicate a stimulatory effect of dibutyryl cGMP (DBcGMP). Thus, although GnRH consistently increases cGMP production in the anterior pituitary, the vast majority of studies demonstrated that LH secretion can be dissociated from increased intracellular concentrations of cGMP. As a result, the exact role of cGMP in GnRH action is still unknown. Obviously, the cyclic nucleotide may mediate other actions of GnRH apart from its stimulation of gonadotropin secretion.

V. Role of Cyclic AMP

The role of cAMP as a mediator of GnRH action is controversial, and the literature is replete with discordant results. Recent studies, however, are beginning to shed some light on the conflicting results. The following discussion on the role of cAMP as a second messenger of GnRH will be subdivided into the following sections: adenylyl cyclase-cAMP system, production of cAMP, GnRH-stimulated activation of adenylyl cylase, effects of cAMP analogs and cAMP-producing agents on LH and FSH release, effects of cAMP analogs / cAMP-producing agents together with GnRH on LH and FSH release and effects of inhibitors of cAMP production on LH and FSH secretion.

i. Adenylyl Cyclase-cAMP System

The generation of cAMP is due to the activation of adenylyl cyclase, and involves receptors, G proteins and the catalytic component. Although the activation of adenylyl cyclase was elucidated for other systems (Cronin et al., 1984), very little is known regarding its activation in pituitary gonadotropes. Briefly, binding of a hormone to a receptor results in the activation of the G proteins and subsequent exchange of GDP for GTP. Two types of G proteins are commonly linked to adenylyl cyclase. These are the stimulatory (G_s) and inhibitory (G_i) proteins (Levitzki, 1986). The binding of GTP to G_s constitutes the on-reaction by which G_s undergoes a conformational change, the α subunit dissociates and increases the sensitivity of adenylyl cyclase for its substrate Mg^{++} ATP. Adenylyl cyclase then catalyses the conversion of Mg^{++} ATP to cAMP and inorganic pyrophosphate (PPi) (Levitzki, 1986 and Gilman, 1987).

Cyclic AMP in turn activates the cAMP-dependent protein kinase (PKA) by binding to its regulatory component, thereby releasing the catalytic component. This free catalytic component is the activated protein kinase, which phosphorylates other proteins. These phosphorylated proteins, in turn, mediate the cAMP-dependent actions of the hormone (Berridge, 1985).

The intracellular concentration of cAMP is not only regulated by adenylyl cyclase, but is also controlled by a cyclic nucleotide phosphodiesterase (PDE) enzyme. PDE converts the active cAMP into an inactive 5'-AMP, thereby terminating the effects of cAMP. It should be mentioned that the activity of some PDEs can be modulated by Ca^{++} working via calmodulin (Conn et al., 1981[a]).

ii. Production of cAMP

While the role of cAMP as a mediator of GnRH action is controversial (Conn et al., 1987[a]), it is commonly accepted that GnRH does stimulate cAMP production in pituitaries of male rats. Hemipituitaries from adult or immature male rats showed an increase in cAMP production in response to GnRH (Borgeat et al., 1972, Makino, 1973, Naor et al., 1975[a], Naor et al., 1978 [a,b] and Bourne and Baldwin, 1987[b]). To the author's knowledge, all studies performed on pituitaries of males did demonstrate an increase in cAMP production. In contrast, early studies using hemipituitaries or dispersed pituitary cells of females were unable to demonstrate a stimulatory effect of GnRH on cAMP production (Naor et al., 1978 [b] and Snyder et al., 1980). Such effects were later demonstrated in pituitaries from female rats (Bourne and Baldwin, 1987[a]), albeit only in the presence of dopamine. Dopamine inhibits PRL secretion (Lamberts and MacLeod, 1990) and adenylyl cyclase activity in mammotropes (Cronin and Thorner, 1982). Since the mammotropes (which comprise a high percentage of pituitary cells in females) are normally under tonic inhibition in vivo, the excision of the anterior pituitaries results in the activation of these cells and increased cAMP production. This, in turn, elevates basal cAMP concentrations. Dopamine inhibits cAMP production in activated mammotropes, reducing a major portion of background cAMP levels, thereby facilitating a better resolution of a GnRH-specific effect on cAMP production (Adams et al., 1979[b]). Presumably, dopamine was not required to demonstrate this action of GnRH in pituitaries of male rats because, as discussed earlier, males have a much lower concentration of mammotropes than females. In short, it appears that GnRH does increase cAMP production in pituitaries of both male and female rats.

iii. GnRH-Stimulated Activation of Adenylyl Cyclase

Initial studies demonstrated that hypothalamic extracts stimulated adenylyl cyclase activity in anterior pituitary homogenates (Jutisz et al., 1972). However, purified GnRH did not activate adenylyl cyclase in pituitary homogenates (Jutisz et al., 1972, Theoleyre et al., 1976 and Berault et al., 1980), or in crude and purified membrane preparations (Clayton et al., 1978 and Berault et al., 1980). Sodium fluoride (NaF), a direct activator of G_S proteins (Gilman, 1987) did activate adenylyl cyclase in these preparations (Jutisz et al., 1972, Deery and Howell, 1973 and Berault, et al., 1980). These discrepancies resulted in the suggestion that the GnRH receptor was disconnected from the site of activation of the catalytic component during the process of homogenization (Jutisz et al., 1972 and Theoleyre et al., 1976), or that the proportion of gonadotropes in the gland was too small to show a significant increase in GnRH-induced adenylyl cyclase activity (Theoleyre et al., 1976). In contrast to these studies, however, Deery and Howell (1973) demonstrated that in the presence of GTP, GnRH caused a marked stimulation of adenylyl cyclase activity in male rat pituitary homogenates, though GnRH in the absence of GTP had little effect on the enzyme activity.

The failure of some investigators to demonstrate adenylyl cyclase activation by GnRH could be due to their experimental protocols. It is now commonly accepted that it is essential to have GTP in adenylyl cyclase assays to obtain hormonal stimulation (Ross et al., 1977 and Abramowitz and Birnbaumer, 1979). However, contaminating amounts of GTP cause a marked increase in the enzyme's basal activity which could mask the effects of the hormone (Birnbaumer et al., 1991). These problems can be easily circumvented by purification of commercially available ATP, as well as the creatine phosphate/creatine phosphokinase reagents used in an ATP-regenerating system (Birnbaumer et al., 1991). Conceivably, the absence of a GnRH effect on adenylyl cyclase activity could be due to contaminating amounts of GTP masking the GnRH-stimulation of the enzyme.

PDE activity is not inhibited by GnRH, (Berault et al., 1980). This, coupled with the fact that GnRH does increase cAMP production in anterior pituitaries (see previous section), and the fact that adenylyl cyclase activation was demonstrated in the presence of GTP (Deery and Howell 1973), suggest that GnRH might increase cAMP production in gonadotropes through the activation of adenylyl cyclase by a GTP-dependent process.

iv. Effects of cAMP Analogs and cAMP-Producing Agents on LH and FSH Release

The direct effects of cAMP analogs on LH release have been studied extensively. Several reports have indicated that administration of dibutyryl cyclic AMP (DBcAMP) and/or 8-bromo-cyclic AMP (8-bromo-cAMP) to hemipituitaries of mature or immature male rats did not stimulate gonadotropin release (Wakabayashi et al., 1973, Naor et al., 1975[a], Naor et al., 1978[b] and Bourne and Baldwin, 1987[b]). These results are at variance with other studies in which DBcAMP stimulated LH and/or FSH secretion from male rat pituitaries (Ratner, 1970, Makino, 1973, Kercret et al., 1977 and Kilpatrick et al., 1977) or dispersed male anterior pituitary cells (Smith et al., 1982).

Various agents, such as pertussis toxin, cholera toxin or forskolin were used to generate cAMP and the effects on LH release were observed. Pertussis toxin (PT) ADP-ribosylates the α subunit of the inhibitory G_i protein. This chemical modification inhibits the binding of GTP to the α subunit, preventing G_i inhibition of adenylyl cyclase (Berridge, 1985). This results in an increase in basal activity of the enzyme and increased cAMP production. Cholera toxin (CT) ADP-ribosylates the α subunit of the stimulatory G_s protein and inhibits GTPase activity (Berridge, 1985). GTP remains bound to the α subunit which then continues to activate the catalytic component and stimulate cAMP formation. On the other hand, forskolin binds directly to the catalytic component and activates the enzyme (Cronin et al., 1984 and Berridge 1985).

Cholera toxin, PGE_2 and PDE inhibitors (theophylline and isobutyl methyl xanthine [IBMX]) were all ineffective in stimulating gonadotropin secretion from pituitaries of males (Naor et al., 1975[a] and Wun et al., 1988) or dispersed male pituitary cells (Conn et al., 1979[a]). These results were obtained in spite of the fact that these agents significantly stimulated cAMP accumulation. The suggestion that these agents could have stimulated cAMP production in cell types other than the gonadotropes, and hence have no effect on LH release, was refuted by the fact that these non-specific drugs do not differentiate between the cell types in the anterior pituitary (Naor et al., 1975[a]).

Incubations of hemipituitaries from females with monobutyryl cAMP (DeKoning et al., 1981) in the presence of theophylline, or dispersed pituitary cells from female rats with DBcAMP or 8 bromo-cAMP, resulted in a slight but significant stimulation of LH and/or FSH secretion (Tang, 1978, Drouin et al., 1978, Conn et al., 1979[a], Tang et al., 1984 and Grotjan and Leveque, 1984). Interestingly, in all of the above situations, the amounts of LH secreted were dramatically less than the amounts secreted in response to GnRH. Moreover, the experimental incubations were always greater than 3 hours.

These reports were later corroborated by perifusion studies which demonstrated a stimulatory effect of cAMP analogs on gonadotropin secretion from proestrous (Turgeon and Waring, 1986[a]) and diestrous II (Bourne and Baldwin, 1987[a]) rat anterior pituitaries. The responses were noteworthy in that the nucleotides appear to elevate basal secretion slightly, but only after a considerable time lag (70 min for proestrous rat pituitaries and 150 min for diestrous II rat pituitaries). Similar results were obtained with cholera toxins and/or forskolin. These agents caused a slight but significant increase in gonadotropin secretion (Conn et al., 1979[a], Cronin et al., 1984 and Turgeon and Waring, 1986[a]) which became apparent after a considerable time lag (Cronin et al., 1984 and Turgeon and Waring 1986[a]).

There appears to be excellent agreement on the effects of cAMP analogs or cAMP-generating agents on gonadotropin secretion from female gonadotropes i.e. cAMP does not exert a dramatic direct effect on gonadotropin secretion, and requires a considerable amount of time before any observed effects are apparent. In contrast, a number of investigators have demonstrated a direct, stimulatory effect of cAMP analogs on gonadotropin secretion from pituitaries of male rats, whereas others have failed to do the same. Unfortunately, it would appear that these differences in findings are almost impossible to reconcile.

Interestingly, these cAMP-induced increases in LH secretion, when observed, were also found to be protein synthesis-dependent, because cycloheximide inhibited the cAMP analog-stimulated LH release (DeKoning et al., 1981).

v. Effects of cAMP Analogs/cAMP-Producing Agents Together with GnRH on LH and FSH Release

Another experimental approach utilized extensively by investigators was the administration of GnRH in the presence of cAMP analogs or cAMP-generating agents as well as PDE inhibitors to determine if these compounds affected GnRH-stimulated gonadotropin secretion. IBMX did not affect GnRH-stimulated gonadotropin secretion from pituitaries of male rats (Wun et al., 1988). Similarly, DBcAMP or IBMX did not affect the GnRH-stimulated LH release from female rat pituitary cells (Conn et al., 1979[a]). In contrast, preincubation of female rat pituitary tissue with monobutyryl cAMP (mbcAMP) and theophylline, DBcAMP or 8-bromo-cAMP augmented the GnRH-stimulated LH release (DeKoning et al., 1981 and Turgeon and Waring, 1986[a]). Significant augmentation required a minimum of 30 min pre-exposure to the cAMP analogs, and greater augmentation was achieved with longer exposures to the cyclic nucleotides (Turgeon and Waring, 1986[a]).

Pretreatment of anterior pituitary cells from female rats with PT, CT or forskolin caused an augmentation of GnRH-stimulated LH release (Cronin et al., 1984). Increases of endogenous cAMP levels by the addition of forskolin caused a 3-fold augmentation of GnRH-induced LH secretion from perifused pituitaries of proestrous rats (Turgeon and Waring, 1986[a]). Exposure to forskolin for longer periods resulted in greater augmentation (Turgeon and Waring, 1986[a]).

The fact that cAMP concentrations were rapidly increased by these agents, but GnRH-stimulated LH release was potentiated only after a considerable time lag, suggested that the cellular mechanisms involving cAMP might be associated with de novo protein synthesis (DeKoning et al., 1981, Cronin et al., 1984 and Turgeon and Waring 1986[a]). Indeed, cAMP-induced augmentation of GnRH-stimulated LH secretion was inhibited by cycloheximide (DeKoning et al., 1981 and Turgeon and Waring, 1986[a]). It appears that cAMP may not be directly involved in gonadotropin secretion, but may affect the process indirectly by stimulating the synthesis of protein(s) that might facilitate or augment the GnRH-induced gonadotropin secretion.

vi. Effects of Inhibitors of cAMP Production on LH and FSH Secretion

Flufenamate (Flu), a trifluoro derivative of N-phenylanthranilic acid (Flower et al., 1980) was shown to inhibit GnRH-stimulated increases in cAMP production in both male and female rat anterior pituitaries (Naor et al., 1975[a,b], Bourne and Baldwin, 1987[a,b] and Bourne, 1988). As a result, this compound was utilized as a pharmacological probe to investigate the potential role of cAMP as a mediator of GnRH-stimulated gonadotropin release. In initial static incubation studies by Naor et al., (1975[b]), flufenamate inhibited the GnRH-stimulated increases in cAMP production, but did not affect the secretion of LH obtained in response to GnRH. These results provided evidence that cAMP can be divorced from LH secretion from male pituitaries.

Perifusion studies, undertaken to investigate potential sex differences in the role of cAMP, indicated that flufenamate completely inhibited the GnRH-stimulated gonadotropin responses from male rat pituitaries, while inhibiting only the second phase of the biphasic secretion from female rat pituitaries (Bourne and Baldwin, 1987 [a,b]). The flufenamate-inhibited, GnRH-stimulated responses were restored in pituitaries from both male and female rats when exogenous DBcAMP was administered in combination with GnRH and flufenamate. The restored response was protein synthesis-dependent and occurred even though the nucleotide by itself did not stimulate gonadotropin secretion from pituitaries of male rats (Bourne and Baldwin, 1987[b]), or caused a marginal increase in basal secretion from pituitaries of female rats (Bourne and Baldwin, 1987[a]). Taken together, the results suggest that the GnRH stimulated gonadotropin secretion in males, and phase II secretion in females, are all indirectly mediated by cAMP, an effect which is associated with de novo protein synthesis. Unfortunately, the discrepancies between the studies using male pituitaries by Naor et al., 1975[b] and Bourne and Baldwin, (1987[b]) cannot be reconciled at present.

vii. Conclusions

It appears that cAMP does play a role as a mediator of GnRH actions. The preponderance of evidence suggests that the nucleotide is not directly involved in gonadotropin secretion, but can influence this process indirectly by activating mechanisms associated with de novo protein synthesis. In this regard, it should be noted that one of the strongest opponents to a role for cAMP (Conn et al., 1987[a]) has recently suggested that the nucleotide can modulate LH secretion (Waters et al., 1990 and Hawes et al., 1991).

VI. Role of Calcium

Calcium is now known to be an important factor in signal transduction mechanisms in numerous tissues (Rasmussen and Barrett, 1984). Its role as a second messenger of GnRH actions is well documented (Conn et al., 1987[b]). The view that Ca^{++} acts as a mediator of GnRH action was consolidated by the demonstration of an increase in intracellular Ca^{++} concentrations (Ca^{++}-mobilization) in gonadotropes within 10 seconds of addition of GnRH (Clapper and Conn, 1985).

i. Calcium Mobilization

The cytosolic Ca^{++} concentration in unstimulated mammalian cells is very low (approximately 0.1 μM). In contrast, the extracellular Ca^{++} concentration is approximately 1 mM (Rasmussen and Barrett, 1984). As a result, a large concentration gradient of Ca^{++} exists across the cell membrane. Activation of mammalian cells is characterized by an immediate increase in cytosolic Ca^{++} concentrations. This increase in cytosolic Ca^{++} concentration can be achieved by two methods.

The first method involves an influx of Ca^{++} from the extracellular medium into the cytosol, down its concentration gradient through activated plasma membrane Ca^{++} channels. The activation of specific plasma membrane Ca^{++} channels by GnRH was suggested by the ability of Ca^{++} channel-blocking agents (verapamil and ruthenium red) to inhibit GnRH-stimulated LH release (Conn et al., 1983 and Conn et al., 1987[a]), and by the LH-releasing action of maitotoxin, a Ca^{++} channel-activating agent (Conn et al., 1987[c]).

Direct evidence for GnRH-activated Ca^{++} channels was obtained from patch-clamp studies of isolated ovine gonadotropes, in which GnRH initiated Ca^{++} currents through the cell membranes (Mason and Waring, 1985, 1986). Further studies indicated that dihydropyridine-sensitive "L"-type Ca^{++} channels are probably involved in GnRH-mediated increases in cytosolic Ca^{++} concentrations (Blotner et al., 1990). Thus, it appears that GnRH activates Ca^{++} channels, causing an influx of extracellular Ca^{++} into gonadotropes. It was also reported that a sustained influx continues as long as GnRH remains bound to its receptor (Bates and Conn, 1984).

The second method of increasing cytosolic Ca^{++} is accomplished by the mobilization of Ca^{++} from intracellular stores. Pioneering studies by Berridge (Berridge and Irvine, 1984 and Berridge, 1987) have demonstrated that various biological systems utilizing Ca^{++} as a second messenger undergo alterations in phosphoinositide turnover as a result of receptor activation. These Ca^{++}-mobilizing hormones activate membrane receptors coupled to guanine nucleotide-binding proteins that facilitate the activation of phospholipase C and ultimately cause the hydrolysis of membrane phosphatidyl inositol 4,5 bisphosphate (PIP_2) into DAG and IP_3 (Abdel-Latif, 1986, Berridge, 1987 and Conn et al., 1987[a]). IP_3 which mobilizes Ca^{++} from intracellular stores (Berridge and Irvine, 1984), is now known to play a pivotal role in increasing cytosolic Ca^{++}.

Studies using rat pituitary cells (Kiesel et al., 1986[b] and Huckle and Conn, 1987) and enriched gonadotrope cell fractions (Andrews and Conn, 1986) have indicated that GnRH stimulates phosphoinositide hydrolysis and phospholipid turnover. Furthermore, GnRH-mediated inositol phospholipid breakdown was shown to be functionally coupled to phospholipase C by a G protein. The phospholipase C-coupled G protein appears to be distinct from that involved in adenylyl cyclase activation (Andrews et al., 1986). In accordance with the activation of phospholipase C, GnRH also stimulated the rapid formation (within 5-10 sec) of IP_3 (Schrey, 1985 and Naor et al., 1986). Furthermore, when permeabilized gonadotropes were treated with IP_3 in the presence of mitochondrial inhibitors and an ATP-regenerating system, a transient increase of cytosolic Ca^{++} was observed (Naor et al., 1988[a]). The Ca^{++} was mobilized (presumably by IP_3) from a non-mitochondrial pool, namely, the endoplasmic reticulum (Naor et al., 1988[a]).

Inositol 1,3,4,5-tetrakisphosphate (IP_4), an intermediate metabolite formed during IP_3 conversion to its inactive isomer inositol 1,3,4-trisphosphate (Berridge, 1987), was implicated as a mediator of Ca^{++} influx in sea urchin eggs (Irvine and Moore, 1986). The fact that GnRH does increase IP_4 levels in pituitary gonadotropes (Morgan et al., 1987) led to the suggestion that IP_4-mediated Ca^{++} entry into gonadotropes may help in the replenishment of the IP_3-sensitive Ca^{++} pool (Naor et al., 1988[a]).

As stated previously, initial studies using enriched gonadotrope cell fractions and the fluorescent Ca^{++} indicator, Quin II, demonstrated that GnRH stimulates a transient increase in cytosolic Ca^{++} concentrations (Clapper and Conn, 1985). It is now apparent that the response consists of rapid oscillations in cytosolic Ca^{++} concentrations, i.e. GnRH causes a biphasic response of cytosolic Ca^{++} concentrations (Naor et al., 1988[a] and Shangold et al., 1988). The response consists of an initial, rapid rise of cytosolic Ca^{++} which lasts for about 8 seconds, before Ca^{++} levels return to basal concentrations. This is followed by a second phase which forms a prolonged plateau (15 min) of elevated cytosolic Ca^{++} (Limor et al., 1987 and Naor et al., 1988[a]). The initial response appears to be due to the mobilization of intracellular Ca^{++}, while the second component consists of extracellular Ca^{++} entering via nitrendipine-sensitive voltage-gated Ca^{++} channels (Chang et al., 1986[a], Limor et al., 1987, Smith et al., 1987, Naor et al., 1988[a] and Shangold et al., 1988).

The conclusion that the initial increase of cytosolic Ca^{++} is the result of mobilization of Ca^{++} from intracellular stores was based on the fact that peak cytosolic Ca^{++} concentrations were reached within 8 sec of GnRH administration in the presence or absence of extracellular Ca^{++} (Naor et al., 1988[a]). The increase in cytosolic Ca^{++} induced by GnRH in a Ca^{++}-free medium accounted for 55% of the maximal increase in Ca^{++} concentrations (Naor et al., 1988[a]). With respect to the generation of IP_3 (and mobilization of intracellular Ca^{++}) in gonadotropes exposed to a Ca^{++}-free medium, it should be noted that the activation of phospholipase C (and hence generation of IP_3) is not dependent on extracellular Ca^{++} (Andrews and Conn, 1986, Naor et al., 1985[a] and Huckle and Conn, 1987). Furthermore, the time course of IP_3 formation by GnRH (5-10 sec) is in good agreement with the observed peak elevation in cytosolic Ca^{++} concentrations (Naor et al., 1986).

The removal of extracellular Ca^{++} or addition of a dihydropyridine Ca^{++} channel blocker (nitrendipine) completely inhibited the secondary rise in cytosolic Ca^{++} concentrations, but did not affect the initial increase (Shangold et al., 1988). Taken together, these observations suggested that the secondary rise in cytosolic Ca^{++} involved an influx of extracellular Ca^{++}.

In summary, the ideas postulated for Ca^{++} mobilization by GnRH in dispersed pituitary cells indicate that GnRH binding to its specific receptors stimulates a rapid hydrolysis of PIP_2. This results in a rapid generation of IP_3 within 5-10 sec. IP_3 mobilizes Ca^{++} from intracellular stores. Ca^{++} mobilized from intracellular stores appears to be responsible for the initial transient increase in cytosolic Ca^{++}. This is followed by a second prolonged phase that appears to be due to an influx of extracellular Ca^{++} through "L"-type Ca^{++} channels.

ii. Calcium and Gonadotropin Secretion

The importance of Ca^{++} in secretory processes was elucidated by Douglas and his colleagues in the course of an extensive series of investigations. Their results led to the concept of "stimulus-secretion coupling", by which increases in cytosolic Ca^{++} in a stimulated cell initiated the secretory process (Douglas and Rubin, 1963). When this concept was later applied to the adenohypophysis, studies were undertaken to manipulate intracellular Ca^{++} concentrations and determine the corresponding effects on gonadotropin secretion.

Elevated concentrations of extracellular K^+ (60 mM) which depolarize cell membranes, activate Ca^{++} channels and allow Ca^{++} to enter gonadotropes, also stimulated gonadotropin secretion (Samli and Geschwind, 1968, Jutisz et al., 1972 and Bourne and Baldwin, 1980). Other agents which elevate cytosolic Ca^{++} and stimulate LH and FSH secretion are: the calcium ionophores (A23187 and ionomycin) which form conduits allowing Ca^{++} to enter the cytosol by moving down transmembrane concentration gradients (Hopkins and Walker, 1978, Conn et al., 1979[b] and Rasmussen and Barrett, 1984) and veratridine, an alkaloid that activates Na^+ channels. The veratridine-induced influx of Na^+ depolarizes the cell and allows Ca^{++} entry into the gonadotropes through Ca^{++} channels (Conn and Rogers, 1980 and Conn et al., 1986). Finally, the administration of lipid-micelles loaded with Ca^{++} elevated cytosolic Ca^{++} concentrations by releasing Ca^{++} intracellularly (Conn et al., 1979[b]).

It should now be evident that elevated cytosolic Ca^{++} concentrations result in gonadotropin secretion i.e. the increased cytosolic Ca^{++} (Ca^{++} mobilization) is coupled to the secretory mechanism(s). The coupling of the Ca^{++} signal to the secretory mechanisms may actually be relayed by at least three distinct mechanisms, a Ca^{++}-calmodulin system, a family of calcium-activated, phospholipid-dependent protein kinases (protein kinase C) and Ca^{++}-dependent, membrane-binding proteins.

iii. The Calcium-Calmodulin System

One of the intracellular targets of Ca^{++} is a Ca^{++}-binding protein known as calmodulin (Means and Dedman, 1980, Rasmussen and Barrett, 1984 and Hartshorne, 1985). Calmodulin is an ubiquitous, heat stable, monomeric, globular protein consisting of 148 amino acid residues with a mass of 17 kDa (Hartshorne, 1985). The molecule is characterized by 2 important structural features. The first is the absence of tryptophan, cysteine and hydroxyproline amino acids (Cheung, 1980). This results in the molecule being able to achieve a high degree of flexibility. The increased flexibility, in turn, facilitates the molecule's ability to interact with effector proteins (Cheung, 1980). The second structural characteristic is the presence of a high proportion of acidic amino acid residues (such as aspartate and glutamate), making calmodulin a highly acidic protein (Cheung, 1980 and Hartshorne, 1985). The carboxylic groups of the side chains of these acidic amino acid residues cluster together to form the Ca^{++}-binding sites.

Calmodulin contains four Ca^{++}-binding domains, termed I, II, III, and IV, starting from the N terminal end (Hartshorne, 1985). Occupancy of any one of the binding sites results in a conformational change in calmodulin. The molecule assumes a more helical conformation (Cheung, 1980 and Means and Dedman, 1980). Additionally, binding of Ca^{++} to any one site increases the affinities of the other sites for Ca^{++} (positive homologous cooperativity) (Rasmussen and Barrett, 1984).

Calmodulin can be considered to be an intracellular receptor for Ca^{++}, because it demonstrates saturable, high affinity, selective and reversible binding with Ca^{++} (Means and Dedman, 1980 and Hartshorne, 1985). Once activated by Ca^{++}, calmodulin binds to and regulates the activities of several enzymes. These enzymes include Ca^{++}-calmodulin-dependent protein kinases, Ca^{++} ATPases, adenylyl cyclase, cyclic nucleotide phosphodiesterases, phospholipase A_2 and myosin light chain kinase (Cheung, 1980, Means and Dedman, 1980, Rasmussen and Barrett, 1984 and Hartshorne, 1985).

The activation of the enzymes by calmodulin begins with the transient increases in cytosolic Ca^{++} (Ca^{++} mobilization). This results in binding of Ca^{++} to the inactive calmodulin (CaM). The formation of the Ca^{++}-calmodulin complex induces a conformational change in the protein, which can then bind to effector enzymes. The formation of the Ca^{++}-calmodulin-enzyme complex results in the activation of the enzyme and subsequent alterations in cellular functions (Rasmussen and Barrett, 1984).

The general sequence of events (Cheung, 1980 and Hartshorne, 1985) may be represented as follows:

(1) $nCa^{++} + CaM \Leftrightarrow Ca^{++}_n . CaM^*$
(inactive) (active)

(2) $Ca^{++}_n . CaM^* + E \Leftrightarrow Ca^{++}_n . CaM^*.E^*$
(inactive) (active calcium-calmodulin-enzyme ternary complex)

(where n represents the number of calcium ions.

CaM represents calmodulin

E represents enzyme

* indicates activated forms of calmodulin or enzyme).

Reductions in the intracellular levels of Ca^{++} cause the dissociation of the ternary complex and loss of enzymatic activity.

It is generally accepted that at least 3 of the 4 Ca^{++}-binding sites on calmodulin must be occupied before calmodulin can activate an enzyme (Rasmussen and Barrett, 1984). However, the exact sequence by which Ca^{++} binds to calmodulin, and calmodulin interacts with the enzymes is dependent on the particular enzyme that is activated (Rasmussen and Barrett, 1984). At present, there are two proposed sequences by which Ca^{++}-calmodulin may activate enzymes. The first scheme deals with the activation of the cyclic nucleotide phosphodiesterase (Huang et al., 1981), while the second scheme represents the activation of Ca^{++} ATPase in red blood cell plasma membranes (Cox et al., 1984).

The first scheme proposed by Huang et al. (1981), describes an ordered sequence of reactions. Positive cooperative binding of the first two Ca^{++} results in the formation of Ca_2^{++}-CaM. This species demonstrates an increased affinity for the phosphodiesterase (PDE) enzyme and binds to PDE. The Ca_2^{++}-CaM-PDE complex is still inactive. However, binding of PDE causes an increased affinity for Ca^{++} of the third and the fourth binding sites on calmodulin. Binding of Ca^{++} to one or both of these binding sites converts the inactive PDE to the active enzyme.

In contrast, the activation of Ca^{++}-ATPase as proposed by Cox et al. (1984) is slightly different. The main difference between this scheme and the former is the fact that the binding of the first three Ca^{++} ions proceeds in a non-cooperative fashion. Binding of the first, second and third Ca^{++} ions to CaM results in the formation of Ca_3^{++}-CaM. This species shows a very high affinity for the Ca^{++}-ATPase (inactive) enzyme and binds to the enzyme to form the activated Ca_3^{++}-CaM-Ca^{++}-ATPase complex. Formation of the activated enzyme ternary complex does not enhance the Ca^{++} affinity for the fourth site on CaM.

iv. Role of Calmodulin in GnRH Action

A role for calmodulin in GnRH action was suggested because the latter causes the redistribution of calmodulin from the cytosol to the plasma membranes (Conn et al., 1981[b]). Calmodulin was also found to be associated with GnRH-receptor patches after GnRH treatment (Jennes et al., 1985). These observations were, however, insufficient to confirm a role for calmodulin in GnRH stimulation of LH release. Such a role for calmodulin was suggested by the use of pharmacological agents (such as pimozide, chlorpromazine and napthalene sulphonamide) which inhibited the activity of Ca^{++}-calmodulin, and blocked LH release in response to GnRH or Ca^{++} ionophores. The fact that the Ca^{++}-calmodulin inhibitors blocked ionophore (A23187 and ionomycin)-stimulated LH release further suggested that Ca^{++}-calmodulin inhibitors were acting at a point after Ca^{++} mobilization (Conn et al., 1981[a]). It should be noted, however, that many of the so-called "calmodulin inhibitors" are not specific and do disrupt other signal transduction processes such as protein kinase C mechanisms (Mori et al., 1980 and Nishizuka, 1984[b]) and those involving the Ca^{++}-dependent, membrane-binding proteins (Pollard et al., 1987). This, in turn, casts some uncertainty on the validity of their use and on the interpretation of data obtained through their use.

v. Protein Kinase C System

a. Activation of Protein Kinase C

As mentioned previously, GnRH-stimulated PIP_2 hydrolysis results in the generation of IP_3 and DAG (Andrews and Conn, 1986). DAG is normally absent in unstimulated cells, but can be produced transiently by the activation of phospholipase C in response to extracellular signals (Nishizuka, 1984[a] and Bell, 1986). Once formed, however, the DAG is rapidly removed (seconds to minutes) as a result of its conversion back to inositol phospholipids by way of phosphatidic acid (PI turnover) and/or further degradation to produce arachidonic acid (Kishimoto et al., 1980 and Nishizuka, 1986). However, the transient appearance of DAG in membranes is associated with the activation of a Ca^{++}-activated, phospholipid-dependent protein kinase commonly referred to as protein kinase C. Increased concentrations of DAG result in the binding of this compound to protein kinase C (PKC), following translocation of PKC from the cytosol to the inner surface of the cell membrane (Nishizuka, 1984[a]). In the presence of the proper mixture of membrane phospholipids (specifically phosphatidyl-serine), DAGs activate PKC by increasing the affinity of the enzyme for Ca^{++}, such that the formation of a Ca^{++}-PKC complex can occur in the absence of any detectable changes in cytosolic Ca^{++} levels (Nishizuka, 1984[b]), or phosphatidyl inositol turnover (Castagna et al., 1982). The Ca^{++}-PKC complex is the activated enzyme which phosphorylates substrate proteins, thereby altering cellular functions (Nishizuka, 1984[a,b] and Conn et al., 1987[a]).

DAGs are the endogenous activators of PKC. However, Castagna et al., (1982) demonstrated that tumor-promoting phorbol esters directly activate PKC in vitro. Apparently these phorbol esters can substitute for DAG, increasing the affinity of PKC for Ca^{++} (Castagna et al., 1982). One important difference in the activation of PKC by phorbol esters (as opposed to the endogenous activator DAG) is that these agents are more potent than DAG since they are not rapidly metabolized (Castagna et al., 1982).

b. Properties of Protein Kinase C

Protein kinase C was first discovered in 1977 by Nishizuka as an undefined protein kinase enzyme which could be activated by limited proteolysis with a Ca^{++}-dependent neutral protease, calpain (Nishizuka, 1988). Later, this enzyme was linked to signal transduction mechanisms by the demonstration of its activation by DAG. PKC is a group of enzymes which catalyze the covalent binding of phosphate groups from ATP to specific substrate proteins (Nishizuka, 1984[a]). The enzymes have a broad substrate specificity and phosphorylate a variety of endogenous proteins, specifically at serine or threonine amino acid residues (Nishizuka, 1984[a]). These residues are normally found adjacent to basic amino acids such as arginine or lysine, on the carboxyl terminal end of substrate proteins (Farago and Nishizuka, 1990).

PKC is widely distributed, and exists as a family of multiple subspecies having closely related structures (Nishizuka, 1988). Initially, four cDNA clones, alpha (α), beta I (βI), beta II (βII) and gamma (γ) were isolated (Nishizuka, 1988). βI and βII are derived from a single RNA transcript by alternative splicing (Kikkawa et al., 1989). Each of these four PKC subspecies consists of a single polypeptide chain (mass 77 kDa) with 4 conserved (C1-C4) and 5 variable (V1-V5) regions (Farago and Nishizuka, 1990). The variable (V) regions account for the differences in the subspecies (Nishizuka, 1988).

The polypeptide chain can be separated into two functionally different components by the Ca^{++}-dependent protease, calpain: one is a hydrophobic domain and the other a hydrophilic one (Nishizuka, 1988). The amino-terminal half, containing regions C1 and C2, forms the hydrophobic domain and is regarded as the regulatory domain of the enzyme (Nishizuka, 1988 and Farago and Nishizuka, 1990). The C1 region contains a tandem repeat of a cysteine-rich zinc-finger-like sequence which probably binds DAG and phorbol esters (Farago and Nishizuka, 1990). The C1 region also facilitates the PKC interactions with the cell membrane (Farago and Nishizuka, 1990). The C2 region, on the other hand, is thought to regulate the Ca^{++}-sensitivity of the enzyme (Nishizuka, 1988, Kikkawa et al., 1989 and Farago and Nishizuka, 1990).

The carboxyl-terminal domain, containing regions C3 and C4, forms the hydrophilic portion of the peptide chain and contains the catalytic domain of the molecule (Nishizuka, 1988, Kikkawa et al., 1989 and Farago and Nishizuka, 1990). Region C3, in particular, forms the ATP-binding site of the enzyme (Nishizuka, 1984 and Farago and Nishizuka, 1990).

Another group of subspecies consisting of delta (δ), epsilon (ε), and zeta (ζ) was also identified (Nishizuka, 1988). This group of subspecies differs from the former group in one aspect, they lack region C2 of the regulatory domain (Nishizuka, 1988; Kikkawa et al., 1989 and Farago and Nishizuka, 1990). Furthermore, the ζ subspecies is lower in mass than the other two, because it is composed of only one zinc-finger-like sequence in region C1 (Nishizuka, 1988 and Farago and Nishizuka, 1990).

Based on the diversity of sequences in the variable region, PKC was separated into several subfractions by chromatography on a hydroxyapatite column (Nishizuka, 1988). These are subfractions type I, II and III, which correspond to γ, β (βI and βII) and α subspecies, respectively. These subspecies all demonstrate subtle differences in their enzymatic properties. The γ or type I subspecies is expressed exclusively in the central nervous system and can be activated by micromolar concentrations of arachidonic acid. This activation by arachidonic acid does not require Ca^{++}, phospholipid or DAG (Kikkawa et al., 1989). PKC with βI and βII sequences (type II) is widely distributed in the brain and other tissues including endocrine tissues like the pituitary (Naor, 1990). This subspecies (type II) shows substantial activity in the absence of added Ca^{++}, as long as DAG and phospholipids are present (Kikkawa et al., 1989). It does not respond well to arachidonic acid (Nishizuka, 1988 and Kikkawa et al., 1989). Finally, PKC with the α sequence (type III) is widely distributed in many tissues and cell types. The α type responds to high concentrations of free arachidonic acid only in the presence of an increased Ca^{++} concentration (Nishizuka, 1988 and Kikkawa et al., 1989). It should be noted that all of the subspecies can be activated by DAG (and phorbol esters) (Nishizuka, 1988 and Parker et al., 1989). The distribution and biochemical properties of the δ, ε, and ζ subspecies are yet to be determined (Nishizuka, 1988 and Kikkawa et al., 1989).

A detailed study by Naor et al.(1988[b]), identified PKC subspecies' distribution in the pituitary. The pituitary gland does not have the γ (type I) subspecies in either soluble or particulate fractions. However, both βI, βII (type II) and α (type III) are present (Naor et al., 1988[b]). Recent studies demonstrated that type II in the pituitary consists mostly of βII enzyme (Naor, 1990).

vi. Role of Protein Kinase C in GnRH Action

Evidence for a role for PKC in GnRH-mediated gonadotropin release was obtained from the demonstration that PKC is present in anterior pituitaries (Turgeon et al., 1984, Naor et al., 1985[b] and Naor, 1990). Moreover, GnRH (or activators of PKC [DAG and phorbol esters]) caused the redistribution of PKC from cytosolic to plasma membrane fractions (Kraft and Anderson, 1983, Hirota et al., 1985, Naor et al., 1985[b] and McArdle and Conn, 1986). Furthermore, the exogenous administration of DAG and phorbol esters stimulated gonadotropin secretion (Smith and Vale, 1980, Conn et al., 1985, Turgeon and Waring, 1986[b], Chang et al., 1986[b], Liu and Jackson, 1987, Stojilkovic et al., 1988, Bourne et al., 1989 and Fahmy et al., 1989).

However, recently, the role of PKC in GnRH-mediated LH release was questioned (McArdle et al., 1987 and Beggs and Miller, 1989). While some researchers suggest a role for PKC in LH release (Stojilkovic et al., 1988, Bourne et al., 1989 and Fahmy et al., 1989), others question its involvement in the secretory process (McArdle et al., 1987 and Conn, 1989). PKC was proposed to be a mediator of other GnRH actions. These include long-term regulation of gonadotrope responsiveness to GnRH by regulating cell surface receptors and/or efficiency of receptor-effector coupling, and gene-expression (Conn, 1989). The controversy surrounding the role of PKC will be addressed in more detail in the "Discussion" section.

vii. Calcium-Dependent, Membrane-Binding Proteins

The process of exocytosis was briefly described earlier. While increased cytosolic Ca^{++} does play a role in this process, the exact mechanisms have remained enigmatic until recently. Elucidation of some of the mechanisms involved in exocytosis was dependent on the discovery of a class of Ca^{++}-binding proteins which interact with biological membranes (Pollard et al., 1987). Synexin, which is an example of this class of proteins, has the ability to cause aggregation of secretory granules and to cause membrane fusion of the aggregated granules and cell membranes into continuous structures (Pollard et al., 1987). Although discovered initially in bovine adrenal medulla, synexin is a widely distributed protein (Pollard et al., 1987).

Synexin is a 47 kDa protein consisting of approximately 180 amino acid residues (Pollard et al., 1987). It is characterized by the presence of lengthy regions of hydrophobicity interspaced by shorter neutral or charged regions. Interestingly, the synexin hydrophobicity patterns resemble most known intrinsic membrane proteins (Pollard et al., 1987).

Under resting conditions when the cytosolic Ca^{++} concentration is low, synexin behaves as a soluble protein by keeping its hydrophobic sequences buried under a hydrophilic shield (Pollard et al., 1987). Upon cell activation, the cytosolic Ca^{++} concentration is increased, resulting in the binding of Ca^{++} to the protein. This causes a conformational change in synexin molecules, whereby the hydrophobic sequences become exposed and hydrophobic interactions then favor polymerization of synexin. The synexin polymers are cigar-shaped rods approximately 10 x 5 nm in size, with each rod consisting of at least 4 synexin molecules. Once formed, the rods associate side by side to form ribbons and clusters (Pollard et al., 1987). It should be emphasized that the role of Ca^{++}

is to initiate polymerization, and the active form of synexin is the polymer (Pollard et al., 1987). The polymerized synexin molecules bind to the charged regions of acidic phospholipids, either on the plasma membrane or secretory granules, and then penetrate into the hydrophobic interior of the phospholipid bilayers (presumably due to its intrinsic membrane protein-like characteristics). In the presence of arachidonic acid (or fatty acids with cis unsaturated double bonds e.g. leukotrienes), the synexin polymers cause aggregation of secretory granules and their fusion with cell membranes into structures similar to those seen in electron micrographs of secreting chromaffin cells (Pollard et al., 1987).

A "hydrophobic bridge" hypothesis was proposed to account for the synexin-mediated fusion process (Pollard et al., 1987). This hypothesis proposed that synexin polymers contact two adjacent membranes simultaneously and form a hydrophobic bridge between the two membranes. Once the bridge is formed, phospholipids on the adjoining surfaces can move across the bridge resulting in their mixing and hence, membrane fusion. The formation of the hydrophobic bridge is believed to act as a driving and directing force for fusion such that the cis and trans monolayers of fusing membranes eventually become continuous, and internal contents are released by exocytosis (Pollard et al., 1987). Whether synexin plays a role in GnRH-mediated gonadotropin release is presently unknown.

A recent report (Burns et al., 1989) suggested that the synexins share a strong structural homology with a group of Ca^{++}-dependent membrane binding proteins known as annexins.

The basic structural features of the annexin family are (i) a highly conserved core domain consisting of 4 or 8 homologous internal repeats of about 70 amino acid residues, and (ii) a nonconserved variable amino-terminal domain (Burgoyne and Geisow, 1989 and Turgeon et al., 1991).

The annexins have one or more Ca^{++}-binding sites. These Ca^{++}-and phospholipid-binding sites are currently believed to be located in the conserved core domain of the molecules (Burgoyne and Geisow, 1989). The non-conserved, variable amino-terminal domain appears to be important in conferring specificity on the functions of each annexin. This domain could also represent a regulatory region, since it contains phosphorylation sites i.e. the annexins are substrates for various tyrosine and serine protein kinases (Burgoyne and Geisow, 1989 and Turgeon et al., 1991).

One of the cellular functions suggested for the annexins is their attachment to cytoskeletal elements and initiation of membrane fusion in exocytosis (Burgoyne and Geisow, 1989 and Turgeon et al., 1991). In this regard, some of the annexins were shown to bind to the membranes of secretory granules, causing their aggregation in the presence of Ca^{++} (Geisow and Burgoyne, 1982 and Drust and Creutz, 1988). Interestingly, the concentration of Ca^{++} required to stimulate granule aggregation and fusion was dramatically reduced in the presence of arachidonic acid (Drust and Creutz, 1988).

Two members of the highly conserved annexin family are present in anterior pituitary cells. These are calpactin I (annexin II) and calpactin II (annexin I) with relative molecular masses (M_r) of 35-36 (Turgeon et al., 1991). In the presence of Ca^{++}, calpactin I (annexin II) was found to associate with anterior pituitary plasma membranes and secretory granule membranes, while calpactin II (annexin I) associated with plasma membranes (Turgeon et al., 1991). This interaction presumably represents an initial event in their ability to promote aggregation of phospholipid vesicles. Annexin I and II also serve as substrates for phosphorylation by protein kinases, including PKC (Turgeon et al., 1991), and as stated earlier, the phosphorylation sites are contained in the amino-terminal tail region. The fact that these proteins are associated with plasma membranes and secretory granule membranes, and also serve as phosphorylation substrates, suggests potential roles in signal transduction and exocytosis in anterior pituitary cells (Turgeon et al., 1991).

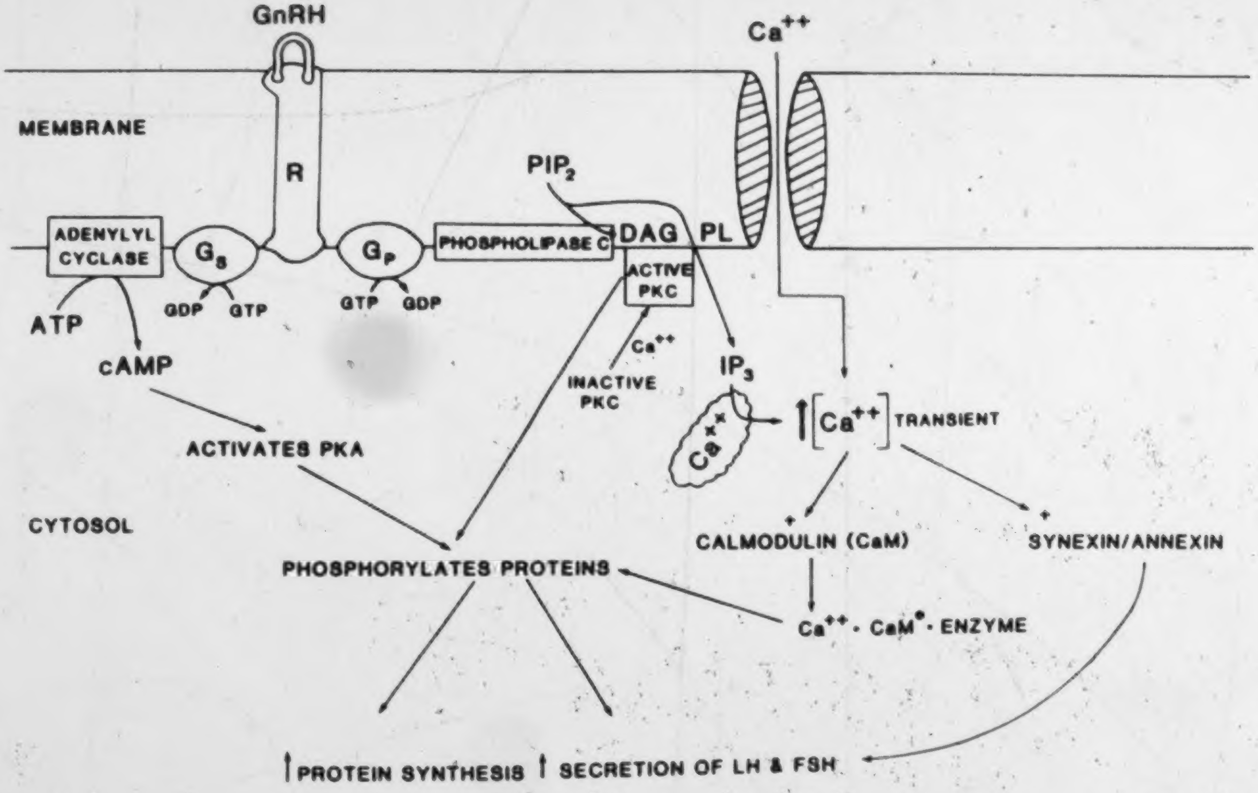
VII. Summary of the Pathway/Roles of Calcium and Cyclic AMP as Mediators of GnRH

The pathways/roles of the second messengers (Ca^{++} and cAMP), which are investigated in the present report are summarized in Fig. 2 (courtesy, Bourne). The alteration of gonadotrope function by GnRH is initiated by binding of the decapeptide to specific receptors on the plasma membranes. GnRH-receptor complex formation is followed by microaggregation of occupied receptors and subsequent activation of G proteins. One of the G proteins, (G_S), activates adenylyl cyclase located on the inner surface of the plasma membrane. This enzyme generates cAMP, which activates cAMP-dependent protein kinases (PKA), resulting in the phosphorylation of proteins.

Phospholipase C, another G protein-activated, membrane-bound enzyme catalyzes the hydrolysis of PIP_2 into DAG and IP_3. IP_3 mobilizes Ca^{++} from the endoplasmic reticulum. GnRH-receptor interactions also activate Ca^{++} channels, initiating an influx of Ca^{++} into gonadotropes. The combined effect of IP_3 and Ca^{++} channel activation is a transient increase in cytosolic Ca^{++} concentrations (Ca^{++} mobilization). This in turn results in the binding of Ca^{++} to calmodulin (CaM) and the activation of a number of enzymes, including the Ca^{++}-CaM-dependent protein kinases which subsequently phosphorylate proteins. Ca^{++} mobilization also activates the Ca^{++}-dependent membrane-binding proteins (synexin and/or annexins) which presumably are involved in exocytosis.

The generation of DAG results in the formation of a PKC.DAG complex following the translocation of the enzyme from the cytosol to the inner surface of the cell membrane. In the presence of membrane phospholipids, PKC's sensitivity to Ca^{++} is increased, resulting in the formation of a Ca^{++}-PKC complex, and subsequent phosphorylation of proteins.

FIG. 2. Schematic representation of the pathways and roles of Ca^{++} and cAMP as mediators of the actions of GnRH. Abbreviations are defined in the text.


GnRH
Ca++
MEMBRANE
R
PIP2
ADENYLYL CYCLASE
Gs
Gp
PHOSPHOLIPASE C
DAG
PL
ACTIVE PKC
GDP
GTP
GTP
GDP
ATP
cAMP
Ca++
INACTIVE PKC
IP3
Ca++
↑[Ca++] TRANSIENT
ACTIVATES PKA
CYTOSOL
+ CALMODULIN (CaM)
+ SYNEXIN/ANNEXIN
PHOSPHORYLATES PROTEINS
Ca++ · CaM* · ENZYME
↑PROTEIN SYNTHESIS
↑ SECRETION OF LH & FSH

The phosphorylated proteins (due to PKC, PKA and CaM) and the activated Ca^{++}-dependent, membrane- binding proteins stimulate gonadotropin secretion, and/or de novo protein synthesis (including the biosynthesis of LH and FSH).

I. Statement of Objectives

It should be apparent that cAMP, Ca^{++} mobilization- and PKC-activated mechanisms have pivotal roles as mediators of GnRH-stimulated gonadotropin secretion. However, their relative contributions to the secretion of gonadotropins, and their interactions during the secretory process remain largely unknown. Consequently, the overall objective of this study was to delineate the roles / interactions and contributions of Ca^{++} mobilization- and PMA-activated mechanisms to the biphasic secretion of gonadotropins from pituitaries of diestrous II rats, as well as the responses obtained from pituitaries of OVX females. Additional studies were also undertaken to investigate the possibility of changes in the roles of cAMP during GnRH-stimulated gonadotropin secretion from pituitaries of OVX rats.

The specific objectives were:

1. To delineate the roles/interactions of Ca^{++} mobilization- and PMA-activated mechanisms as mediators of the biphasic gonadotropin secretion from pituitaries of diestrous II rats.
2. To determine whether removal of the ovaries would cause a change in the roles / interactions of the Ca^{++} mobilization- and PMA-activated mechanisms.
3. To determine whether ovariectomy would cause a change in the role of cAMP as a mediator of GnRH-stimulated gonadotropin secretion.

J. Rationale for Experimental Models

The animals chosen as pituitary donors for the initial objective were females in the diestrous II stage of the estrous cycle. This choice was based on the fact that the biphasic secretions of gonadotropins from pituitaries of diestrous II rats were previously characterized in some detail (DeKoning et al., 1976, Bourne and Baldwin, 1980, Baldwin et al., 1983 and Bourne and Baldwin, 1987[a]). Thus, the use of diestrous II females maintained a consistency of the animal model, ensuring appropriate comparisons of the present data with previous observations. Short-term, OVX animals were used for the second and third objectives because of time (and cost) considerations. An ovariectomized period of 72 h was chosen because previous studies had demonstrated this to be a sufficient period for the clearance of most of the serum gonadal steroids (cf. Baldwin et al., 1983), and dramatic changes in the secretory profiles of LH and FSH were already established by this time (Baldwin et al., 1983 and Bourne et al., 1988, 1989).

A dynamic perifusion system was chosen as the experimental model system, because it facilitates modification of the medium without tissue manipulations, and prevents the accumulation of proteolytic enzymes, metabolic by-products and other anterior pituitary hormones which conceivably could exert destructive and/or paracrine feedback effects on the anterior pituitary cells. The dynamic characteristics of the system also ensure a continuous replenishment of nutrients and secretagogues, maintaining constant concentration gradients, which facilitates the diffusion of nutrients/secretagogues to the cells. Furthermore, the basal secretions of LH and FSH obtained prior to the administration of secretagogue(s) serve as controls for comparison with the secretion obtained in response to the secretagogue(s). In other words, each perifusion chamber effectively serves as its own control. The system is also very simple in design, easy to use and generates highly reproducible results (cf. Appendix 3).

A tissue perifusion system was used instead of a dispersed pituitary cell perifusion system because as discussed earlier, the tissue perifusion system duplicates in vivo secretory profiles and phenomena, (Edwardson and Gilbert, 1975, Bourne and Baldwin, 1980, Waring and Turgeon, 1980 and Baldwin et al., 1983), while dispersed pituitary cells do not (Naor et al., 1982, Catt et al., 1983 and Chang et al., 1987). These observations afford some degree of confidence that the system used in the present study duplicates the cellular mechanisms which are operative in vivo.

Chapter III

MATERIALS AND METHODS

A. Materials

The materials used in the study were as follows: Biogel P-100 (100 - 200 mesh [wet]) (Bio-Rad, Mississauga, Ontario); Eagle (modified) minimum essential amino acids without glutamine, Eagle (modified) minimum essential vitamins (Flow Laboratories, Mississauga, Ontario); GnRH (Peninsula, Belmont, CA and Calbiochem, La Jolla, CA); ionomycin (calcium salt) (Calbiochem, La Jolla, CA); Millipore Sterivex-GS filters (Millipore, Bedford, MA); sodium iodide (NaI^{125}) (Radiopharmacy Laboratory, University of Alberta, Edmonton, Alberta and Amersham, Oakville, Ontario) and Sephadex G-75 (Pharmacia Fine Chemicals, Dorval, Quebec).

Materials purchased from Fisher Scientific Company, (Don Mills, Ontario) were: calcium chloride, chloramine T, ethyl ether, magnesium sulphate, potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate, sodium chloride, sodium hydroxide, sodium phosphate monobasic and sodium phosphate dibasic.

Adenosine 5'-monophosphate (5'-AMP) ([Type II] sodium salt), bovine serum albumin (BSA) (RIA grade, for radioimmunoassays and fraction V, for coating tubes), n-butyric acid (sodium salt), cycloheximide, N^6,2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate (DBcAMP), dopamine (3-hydroxy tyramine), ethylene diamine tetraacetic acid (EDTA) (tetra sodium salt), flufenamic acid (Flu), gelatin (Type I), α D (+) glucose, isobutyl methyl xanthine (IBMX), phorbol 12-myristate 13-acetate (PMA), sodium fumerate, sodium glutamate and sodium pyruvate, were all purchased from Sigma Chemical Company, (St. Louis, MO).

GnRH was prepared as a stock solution (1 mM) in 0.154 M sodium chloride solution and stored in aliquots at -70°C. On the day of an experiment, GnRH was diluted with the perifusion medium to obtain the final concentrations used.

Ionomycin was dissolved in 95% ethanol and stored as a stock solution (1 mM) at 5°C. Aliquots of the ionophore were diluted with the perifusion medium to obtain the final concentrations used. PMA was prepared as a concentrated stock solution (1 mM) in an acetone/ethanol mixture following the procedures of Prince et al., (1973), and diluted with the perifusion medium on the day of an experiment. Flufenamic acid was dissolved in 0.1 N sodium hydroxide solution. Aliquots were added to the perifusion medium before adjusting the pH. All vehicles' concentrations (0.1% v/v) had no measurable effect on gonadotropin secretion.

Cycloheximide, dopamine and IBMX were weighed and added directly to the perifusion medium prior to the addition of sodium bicarbonate solution. DBcAMP, butyrate, and 5'-AMP were also solubilized in the perifusion medium directly.

The light-sensitive chemicals (e.g. ionomycin and PMA) were shielded from light by wrapping all containers and medium reservoirs with aluminum foil.

B. Methods

I. Animals and Tissue Preparation

Adult, female (50 to 60-days-old) Sprague-Dawley rats were obtained from Bioscience Animal Services (University of Alberta, Edmonton, Alberta), or were bred from a colony maintained by the Department of Physiology (University of Saskatchewan, Saskatoon, Saskatchewan). The animals were housed in a temperature- and light-controlled environment (20 ± 1°C, lights on 0600-1800 h) with food and water supplied ad libitum. Depending on the objectives of the experiments, regularly cycling diestrous II rats exhibiting at least two or more consecutive 4-day estrous cycles (determined by daily vaginal cytology), or OVX (72 h) females were used as the pituitary donors. Bilateral ovariectomies of randomly cycling female rats were performed under light ether anesthesia as follows: a small transverse incision was made on the lower back. The ovaries along with part of the fallopian tubes were then excised through bilateral incisions of the musculature. The incisions in the musculature were sutured with surgical silk (size 4-0), while the skin incisions were closed with surgical wound clips.

On the day of an experiment, unanesthetized animals were killed by decapitation at 1200 h standard time, and the pituitaries excised. The posterior pituitaries were removed, and the anterior pituitaries placed in prewarmed perifusion medium (37°C) to minimize temperature shock (Bourne and Baldwin, 1980).

II. Perifusion Medium

Kreb's Improved Ringer Bicarbonate (KIRB) solution (McKenzie and Dawson, 1969) was used as the perifusion medium (cf. Appendix 1). The medium was supplemented with minimum essential amino acids (without glutamine) and vitamins. The inorganic components (sodium chloride, potassium chloride, calcium chloride, potassium dihydrogen phosphate and magnesium sulphate) and organic components (sodium pyruvate, sodium fumerate, sodium glutamate and glucose) of the medium were prepared and stored as stock solutions. On the day of an experiment, the sodium bicarbonate solution was prepared and gassed with CO_2 for 1 h. While the bicarbonate solution was being gassed, frozen aliquots of the vitamins and amino acids were allowed to thaw and the perifusion medium was prepared. Appropriate volumes of the inorganic solutions were added first, followed by the organic solutions, vitamin and amino acid aliquots. The sodium bicarbonate was added in the final step, and the medium mixed well. For those experiments requiring cycloheximide, the cycloheximide was added directly to the perifusion medium before the addition of the sodium bicarbonate solution (thus, the anterior pituitaries were exposed to cycloheximide in the KIRB medium as soon as they were excised). In the case of flufenamate, aliquots of sodium flufenamate were added to the

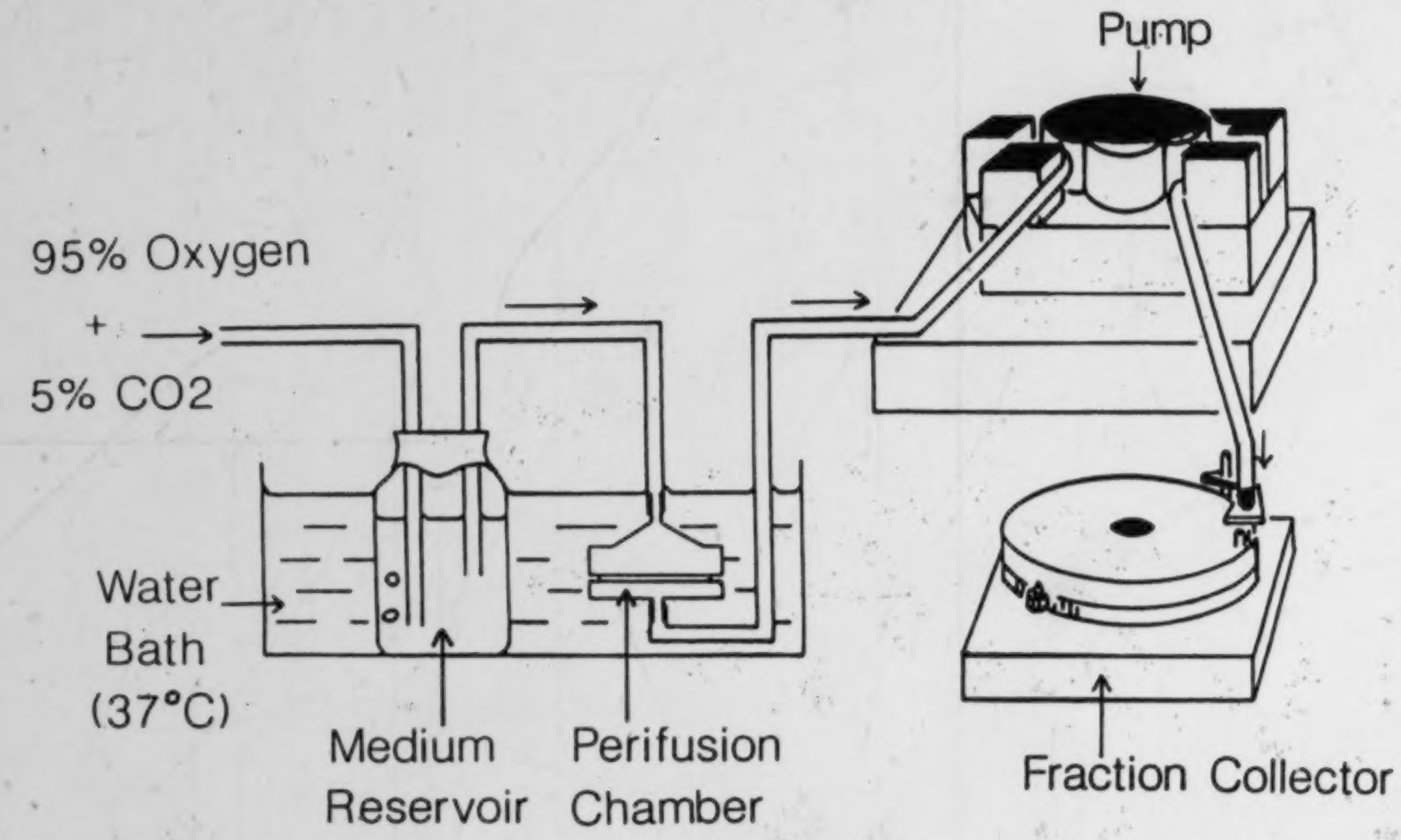
KIRB medium after the addition of the bicarbonate solution, (the pituitaries were also exposed to flufenamate as soon as they were excised). After all the components were added, the perifusion medium was gassed with a mixture of 95% O_2 / 5% CO_2 for 10 minutes, the pH adjusted to 7.4 with 10% NaOH at room temperature, and cold sterilized through Millipore Sterivex-GS (0.22 µm) filters. The medium was then aliquoted into sterile fleakers which served as medium reservoirs.

III. The Perifusion System

A dynamic, anterior pituitary tissue perifusion system (Bourne and Baldwin, 1980) was used for the experiments. A schematic diagram of the system is shown in Fig.3 (courtesy, Bourne). The perifusion chambers (Millipore Swinnex-13 filter holders), associated tubings and all glassware were autoclaved before use. The medium reservoirs were placed in a waterbath at 37°C and gassed continuously with 95% O_2 / 5% CO_2. The perifusion chambers were initially filled with the medium, immersed in the waterbath and allowed to equilibrate at 37°C for 1 h, during which time the pituitaries were collected. Two excised, anterior pituitaries were cut into quarters, and placed into a perifusion chamber, using a short-tipped Pasteur pipette. Air bubbles were expelled from the system by reversing the direction of flow of the medium, while finger flicking the perifusion chambers. Three or six chambers were run simultaneously.

A multichannel peristaltic pump (Minipuls-2, Gilson) was used to maintain a flow rate of 0.25 mL/min. The perifusate from the first hour was discarded because of high, non-specific release of gonadotropins due to tissue handling and manipulations (Bourne and Baldwin, 1980). Sequential effluent fractions were collected at room temperature every 10 minutes in 2% (w/v) BSA-coated tubes placed in Redirac fraction collectors (LKB BROMMA model 2112). The samples were collected at room temperature, since previous studies demonstrated no significant changes in LH immunological activity in aliquots of

FIG. 3. Schematic diagram of the anterior pituitary tissue perifusion system. A waterbath was used to maintain the medium reservoirs and the perifusion chambers at 37°C. Krebs Improved Ringer I medium was delivered at a rate of 0.25 mL/min to the perifusion chambers by a Gilson peristaltic pump. Sequential effluent fractions were collected at room temperature at 10 min intervals using LKB Redirac fraction collectors. The medium was continuously gassed with 95% O_2 / 5% CO_2 mixture throughout the experimental period.


Pump
95% Oxygen
+
5% CO2
Water
Bath
(37°C)
Medium
Reservoir
Perifusion
Chamber
Fraction Collector

samples frozen immediately after collection vs. those aliquots which were collected at room temperature for the duration of the experiment (Bourne and Baldwin, 1980).

Test compounds were dissolved in the perifusion medium and introduced into the chambers by (1) stopping the pump and the timer of the fraction collector, (2) quickly transferring the inlet tubings from the control fleaker to the fleaker containing the test compounds and (3) restarting the pump and the timer of the fraction collector simultaneously. At the end of an experiment, the samples were stored frozen (-20°C) until assayed for LH and FSH.

IV. Radioimmunoassay of LH and FSH

The samples were assayed for LH and FSH using the double-antibody radioimmunoassay (RIA) kits from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). All samples were assayed in duplicate, and those from a given series of experiments were assayed at the same time.

i. Iodination Procedure

The gonadotropins were iodinated with I^{125} using a modified version of the chloramine T method described by Hunter and Greenwood, (1962). Briefly, 40 MBq of radioactive NaI^{125} was added to 5 µg of highly purified hormone (NIDDK-rLH-I-7 or NIDDK-rFSH-I-7). The reaction was initiated by the addition of 25 µL chloramine T (0.5 mg/mL), and the reactants were mixed by finger-flicking the test tube which served as the reaction vessel. The reaction was allowed to proceed for 90 s and was stopped by the addition of 200 µL of 0.05 M EDTA-PBS-1% (w/v) BSA (pH 7.5) solution. The entire contents of the reaction vessel were then transferred to a Sephadex G-75 column which was used to separate the labeled hormone, unlabeled hormone and free iodine.

The Sephadex column was prepared as follows: 1 g of Sephadex G-75 gel was washed 3 times with 0.05 M sodium phosphate buffer (pH 7.5) and allowed to degas overnight under a vacuum. The following day, a 10 mL disposable pipette was carefully packed with the gel. Just before starting the iodination, 3 mL of 0.05 M EDTA-PBS-1% (w/v) BSA was run through the column to reduce non-specific binding. The contents from the iodination procedure were eluted from the column with 0.05 M EDTA-PBS-1% (w/v) BSA (pH 7.5). Twenty fractions (5 drops each) were collected in 12 x 75 mm tubes containing 0.1 mL of PBS-0.1% (w/v) gelatin (pH 7.0). The tubes were counted in a gamma counter (Micromedic Systems, 4/200 Automatic Gamma Counter) and the iodinated peaks were pooled. Aliquots of 30×10^6 cpm were stored frozen at -70°C until required.

ii. Purification of Iodinated Hormones

One day before the assay, the iodinated hormone was purified using a Biogel P-100 column. Preparation of the column involved the following procedures. Four g of Biogel-P-100 was washed with distilled water and allowed to swell and degas overnight under vacuum. The following day, the Biogel was kept at 4°C for several hours before packing the column (in a cold room at 4 to 8°C). The column was then washed with 40 mL of 0.01 M sodium barbital, 0.15 M sodium chloride solution (pH 7.6). Prior to use of the column for the first time, 5 mL of 0.01 M sodium barbital, 0.15 M sodium chloride (pH 7.6), containing 2% (w/v) BSA was eluted with 80 mL of 0.01 M sodium barbital, 0.15 M sodium chloride solution. All gel permeation procedures using the Biogel P-100 column were performed in the cold room.

On the day of purification, the iodinated hormone was added to the column, allowed to enter the gel, and eluted with 0.01 M sodium barbital, 0.15 M sodium chloride solution (pH 7.6). Thirty fractions (14 drops each) were collected, counted and the peak tubes were pooled. The iodinated peaks were diluted to 10,000 cpm/100 µL with PBS-0.1% (w/v) gelatin solution (pH 7.0) for use in the assays.

iii. Radioimmunoassay Procedure

A 3-day RIA protocol was used for these studies (couirtesy, Baldwin). The assays were performed using 12 x 75 mm borosilicate tubes and the reagents were added using a Micromedic automatic pipetting station. On the first day of the assay, the buffer (PBS-0.1 % (w/v) gelatin, pH 7.0), hormone for the standard (NIDDK-rLH-RP2 used in Edmonton and NIDDK-rLH-RP3 in Saskatoon or NIDDK-rFSH-RP2 in both cities), samples, radioiodinated hormone and antiserum (NIDDK-anti-rLH-S10/NIDDK-anti-rFSH-S11) were added simultaneously and incubated at room temperature for 24 h. On the second day, anti-rabbit gamma globulin (ARGG) was added to the tubes and incubated at 4°C for an additional 24 h period. On the third day, 1.5 mL of phosphate-buffered saline (pH 7.0) was added to the tubes which were centrifuged at 2190 x g at 4°C for 45 minutes. At the end of the centrifugation, the supernatants were aspirated and the precipitates were counted for 2 minutes in the gamma counter.

iv. Estimation of Sample Concentrations of LH and FSH

The sample estimates of the assays were calculated using the log-logit transformation and weighted least-square regression analysis as described by Rodbard et al. (1969). Samples of the standard curves are shown in Appendix 2. The values were compared to NIDDK-rLH-RP2 (Edmonton) / NIDDK-rLH-RP3 (Saskatoon) and NIDDK-rFSH-RP2. The NIDDK-rLH-RP2 is 61 times more potent than NIDDK-rLH-RP1, while, NIDDK-rLH-RP2 and NIDDK-rLH-RP3 are of equal potency. Consequently, the sample estimates of LH in this study are 61 times lower than those compared to NIDDK-rLH-RP1. Similarly, the estimates using NIDDK-rFSH-RP2 are 45 times lower than those obtained through the use of NIDDK-rFSH-RP1.

The LH and FSH intra- and interassay coefficients of variation were calculated from estimates of pooled samples. The formula used to calculate the coefficients of variation is:

Coefficient of variation = (standard deviation/mean) X 100

The intraassay and interassay coefficients of variation for LH were 7.7% and 9.7%, respectively, while those for FSH were 10.8% and 13.1%, respectively.

V. Analysis of Perifusion Data

The total amounts of the gonadotropins released above basal levels in response to continuous infusions of secretagogues were calculated by: (i) determining the mean basal secretion per 10 min from the amounts of hormones released prior to the administration of secretagogues (ii) subtracting the mean basal secretion per 10 min from the amounts of hormones in each of the 10 min fractions collected during the 4 h experimental period, and (iii) summing the amounts of hormones released in each 10 min fraction from the time of infusion of the secretagogue up to their termination. The data were statistically analyzed by performing one-way analysis of variance, and differences between means were determined by Duncan's Multiple Range test using the Number Cruncher Statistical System (NCSS, Kaysville, UT). In those instances where secretagogues did not stimulate gonadotropin secretion above basal levels, a value of zero was used for statistical analysis. A value of $p<0.05$ was considered to be statistically significant.

The cumulated amounts of LH and FSH released (above the basal levels) were calculated by the sequential addition of the total amounts of gonadotropin released at each 10 min interval. These values were plotted as a function of time, and the slopes ("mean" secretion rates) were calculated by linear regression analysis. Multiple linear regression analysis using the BMDP statistical software available on the SKYFOX network of the VAX/VMS mainframe computer system (University of Saskatchewan) was utilized to determine whether the slopes of the regression lines were different. A value of $p < 0.05$ was considered statistically significant. When significant differences in the slopes were found, different straight lines were fitted to the data. When the slopes were not significantly different a single straight line was generated.

VI. Determination of Pituitary cAMP Production

The static incubation system described previously by Naor et al. (1975 [a]) was used to determine the effects of GnRH and/or flufenamate on cAMP production in pituitaries obtained from OVX rats. Following decapitation and excision of anterior pituitaries from OVX rats, the pituitaries were cut into halves, and paired hemipituitaries were placed into flasks (equivalent of 1.5 pituitaries/flask). The hemipituitaries were preincubated in 1 mL of KIRB medium containing 10 µM of dopamine ± IBMX (0.5 mM) for 2 h in a Dubnoff metabolic shaker at 37°C under an atmosphere of 95% O_2 / 5%CO_2. After the preincubation, the medium was quickly changed and the hemipituitaries were incubated for an additional 2 h in KIRB medium in the presence or absence of GnRH and/or flufenamate. Following the experimental incubations, the medium was removed and stored frozen at -20°C, until assayed for LH. The hemipituitaries were placed in 1 mL of boiling sodium acetate buffer, pH 6.2 (the buffer used in the cAMP assay) and homogenized. The homogenates were then transferred to polypropylene tubes and placed in a boiling water bath for 10 minutes, after which time the homogenates were centrifuged at 2190 x g for 15 minutes. Finally, the supernatants were removed and stored frozen at -70°C until assayed for cAMP on the following day.

VII. Radioimmunoassay of cAMP

The samples were assayed for cAMP in duplicate using a New England Nuclear (Montreal, Quebec) cAMP radioimmunoassay kit. Included in the kit were sodium acetate buffer (pH 6.2), cAMP standard, cAMP antiserum complex, radioiodinated cAMP, cAMP carrier serum complex and a cAMP precipitator.

On the first day, appropriate volumes of assay buffer, standards or unknown samples, radioiodinated cAMP and cAMP antiserum complex were added to numbered 12 x 75 borosilicate glass test-tubes, mixed well and incubated at 4°C for 16-18 h. On the second day, 0.5 mL of the cAMP precipitator was added to all tubes. The contents of the tubes were thoroughly mixed, and centrifuged at 2190 x g for 45 minutes at 4°C. Following centrifugation, the supernatants were aspirated and the pellets counted for 2 minutes in the gamma counter. Sample estimates were then calculated using the log-logit transformation and weighted least square regression analysis (as above).

VIII. Analysis of Data

The amounts of LH secreted and cAMP produced from pituitaries in experiments using the static incubation system were statistically analyzed by Student's t-test for matched pair groups. A value of $p<0.05$ was considered significant.

Chapter IV

RESULTS

A. Effects of Ionomycin and PMA on Gonadotropin Secretion from Pituitaries of Diestrous II Rats

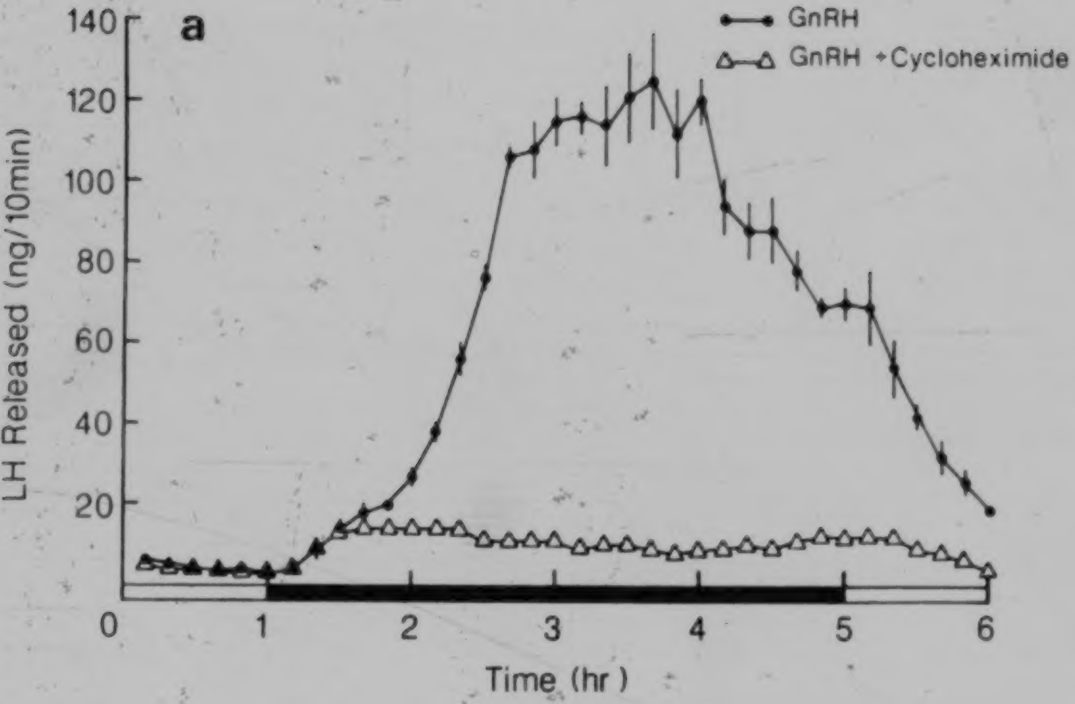
As stated earlier, GnRH-stimulated gonadotropin secretion from pituitaries of intact, female rats is characterized by a biphasic response. Studies undertaken to define the role of extracellular Ca^{++} in mediating the biphasic secretion of LH resulted in the separation of the response into three components (Bourne and Baldwin, 1980): the phase I secretion was shown to be dependent on extracellular Ca^{++}, while the second phase secretion was dissociated into a relatively large, extracellular Ca^{++}-dependent component, and a smaller, extracellular Ca^{++}-independent component (Bourne and Baldwin, 1980).

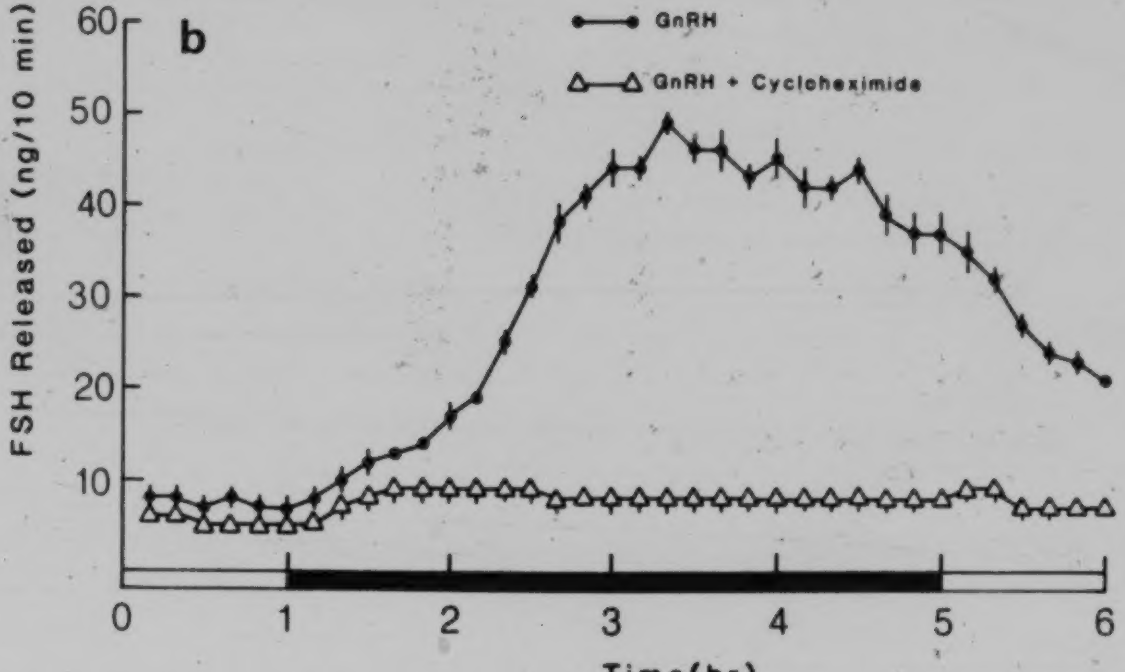
Additionally, a number of studies have suggested that LH release may be a composite of Ca^{++} mobilization- and PKC-activated mechanisms (Harris et al., 1985, Naor and Eli, 1985, Davidson et al., 1987, Naor et al., 1987 and Wooge and Conn, 1987). Moreover, in other endocrine systems, mechanisms activated by Ca^{++} mobilization were shown to regulate the initial, secretory responses, while PKC appeared to mediate the later, sustained responses (Kojima et al., 1983 and Zawalich et al., 1983). These observations, coupled with the results of a previous report which implicated PKC as a potential mediator of the phase II, extracellular Ca^{++}-independent component of gonadotropin secretion (Bourne et al., 1989), suggest that Ca^{++} mobilization might be involved with the extracellular Ca^{++}-dependent components of the biphasic LH secretion, whereas PKC might mediate the extracellular Ca^{++}-independent, second phase release of the hormone.

Consequently, the Ca^{++} ionophore, ionomycin, was used to mobilize calcium without activating PKC (Rasmussen and Barrett, 1984), while the phorbol ester, PMA was used to activate PKC, in an attempt to delineate the contributions of Ca^{++}-mobilization and PMA-activated mechanisms towards the biphasic secretion of LH and FSH.

The biphasic secretion of LH and FSH obtained from pituitaries of diestrous II rats in response to GnRH was previously characterized by Bourne and Baldwin (1980). Initially, dose-response curves using various concentrations of GnRH were constructed, and 1 nM GnRH (a submaximal concentration) was chosen as the experimental dose (Bourne and Baldwin, 1980). The results of initial experiments conducted to illustrate the characteristics of gonadotropin secretory profiles from pituitaries of diestrous II rats in response to continuous infusions of 1 nM GnRH are shown in Fig.4[a,b]. The response was characterized by an initial, small release of the hormone (phase I), followed approximately 60 min later by a greatly augmented secretion rate (phase II). In agreement with previous reports (Bourne and Baldwin, 1980, 1987[a]), 5 μM cycloheximide inhibited the phase II secretion without appreciably affecting the phase I secretion (Fig.4[a,b]), i.e. cycloheximide can be used to differentiate between phase I and phase II secretions. Consequently, cycloheximide was used in subsequent experiments to determine whether ionomycin-and/or PMA-induced gonadotropin secretion represents phase I (protein synthesis-independent) or phase II (protein synthesis-dependent) mechanisms. The concentration of cycloheximide used in this study was based on the fact that this was the lowest dose which inhibited the in vitro protein synthesis-dependent self-priming effect of GnRH, without affecting the initial response (Edwardson and Gilbert, 1975). Higher concentrations which completely inhibited GnRH-stimulated protein synthesis (Liu and Jackson, 1978) also inhibited the GnRH-stimulated augmented phase of the biphasic response obtained from pituitaries of diestrous II rats without affecting the initial response (Dekoning et al., 1976). While the secretory profiles of FSH qualitatively resembled the LH profiles (Fig 4[a,b]), the magnitude of the FSH response was smaller than the LH response.

FIG. 4. Responses from pituitaries of diestrous II rats to continuous infusion (4 h) of 1 nM of GnRH (indicated by the black bar) in the absence or presence of 5 μM of cycloheximide. Cycloheximide was present in the medium from the beginning of the experiment. Results are the means ± SEM (n=3). Absence of standard error bars is indicative of the fact that SEMs are smaller than the size of the symbol. Secretory profiles for LH are shown in the upper panel marked **a**, whereas those for FSH are depicted in the lower panel marked **b**. Abbreviations are defined in the text.


a
GnRH
GnRH +Cycloheximide
LH Released (ng/10min)
140
120
100
80
60
40
20
0
1
2
3
4
5
6
Time (hr)


b
GnRH
GnRH + Cycloheximide
FSH Released (ng/10 min)
60
50
40
30
20
10
0
1
2
3
4
5
6
Time(hr)

Cumulated amounts of LH and FSH released in response to GnRH were plotted as a function of time to demonstrate the biphasic nature of the gonadotropin response. As shown in Fig.5[a,b] there are two distinct slopes, indicating two distinct "mean" rates of LH and FSH secretion. The "mean" phase I secretory rates (15.3 ng/10 min [LH]; 6.5 ng/10 min [FSH]) were significantly different from the "mean" secretory rates for phase II (100.7 ng/10 min [LH]; 34.9 ng/10 min [FSH]), ([$p<0.001$] for LH and FSH).

The rationale of expressing the data in this form is merely to demonstrate significant differences in the "mean" secretory rates of the gonadotropins in the two phases, and thus indicate a biphasic response. This approach allows the author to make a judgement on the expression of a biphasic response based on statistics. As a result, not much importance is given to the absolute values of the "mean" secretory rates, except in their use to indicate significant changes in the secretory rates of phase I and II.

The next series of experiments was performed to determine an experimental dose of ionomycin. As shown in Fig.6, 1 μM ionomycin did not stimulate LH secretion above basal levels until the last hour of secretagogue infusion, when it resulted in a small amount of LH secretion. However, the 10 μM ionomycin-induced LH secretion was characterized by an increased, low rate of gonadotropin secretion, which reached a maximum level approximately 2 h from the start of the ionomycin infusion. The secretion was maintained even after the termination of infusion of the secretagogue (Fig.6 and Fig.7[a]). Similar characteristics were observed for FSH (Fig.7[b]). Interestingly, the ionomycin (10 μM) - induced responses were qualitatively similar to the secretory profiles of LH and FSH obtained in response to GnRH in the presence of cycloheximide, i.e., they resembled the phase I secretion (cf. Figs.4[a,b] and Fig.7[a,b]). Additionally, the magnitude of the responses was similar (cf. Fig.11[a,b]). These considerations formed the basis for the decision to use 10 μM ionomycin in all subsequent studies.

Cycloheximide exerted a significant ($p<0.05$) effect on the ionomycin-induced LH response (Fig.7[a]), suggesting that the ionomycin-stimulated LH secretion is only partially

FIG. 5a. Cumulated amounts of LH released from pituitaries of diestrous II rats in response to continuous infusion (4 h) of 1 nM of GnRH. Each point represents the total amount of LH released up to that time during the 4 h of GnRH administration. The slopes calculated from linear regression analysis (see "Materials and Methods") were used to calculate the "mean" secretory rates for phase I and II. The slope for secretion occurring in the first hour indicates the "mean" secretory rate for phase I (15.3 ng/10 min), while the remaining slope represents the "mean" secretory rate for phase II secretion (100.7 ng/10 min). The correlation coefficient for the line fitted to the data for phase I secretion was 0.9903, while the value for phase II was 0.9967.

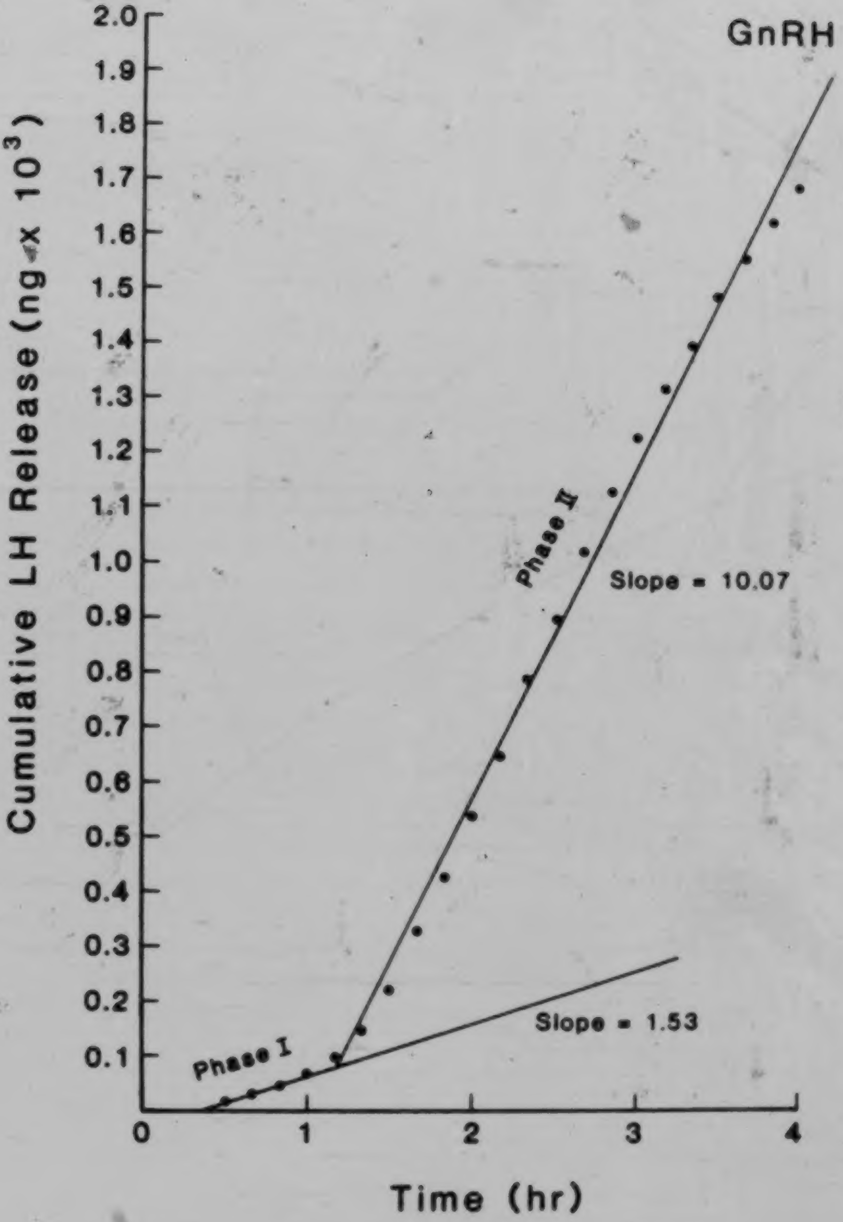

GnRH
Cumulative LH Release (ng x 10³)
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
Time (hr)
Phase I
Phase II
Slope = 10.07
Slope = 1.53

FIG. 5b. Cumulated amounts of FSH released from pituitaries of diestrous II rats in response to continuous infusion (4 h) of 1 nM of GnRH. The "mean" secretory rate for phase I is 6.5 ng/10 min, while the value for phase II is 34.9 ng/10 min. The correlation coefficients for the lines fitted to the data for phase I and phase II are 0.9927 and 0.9988, respectively. For further details, see legend for Fig. 5a.

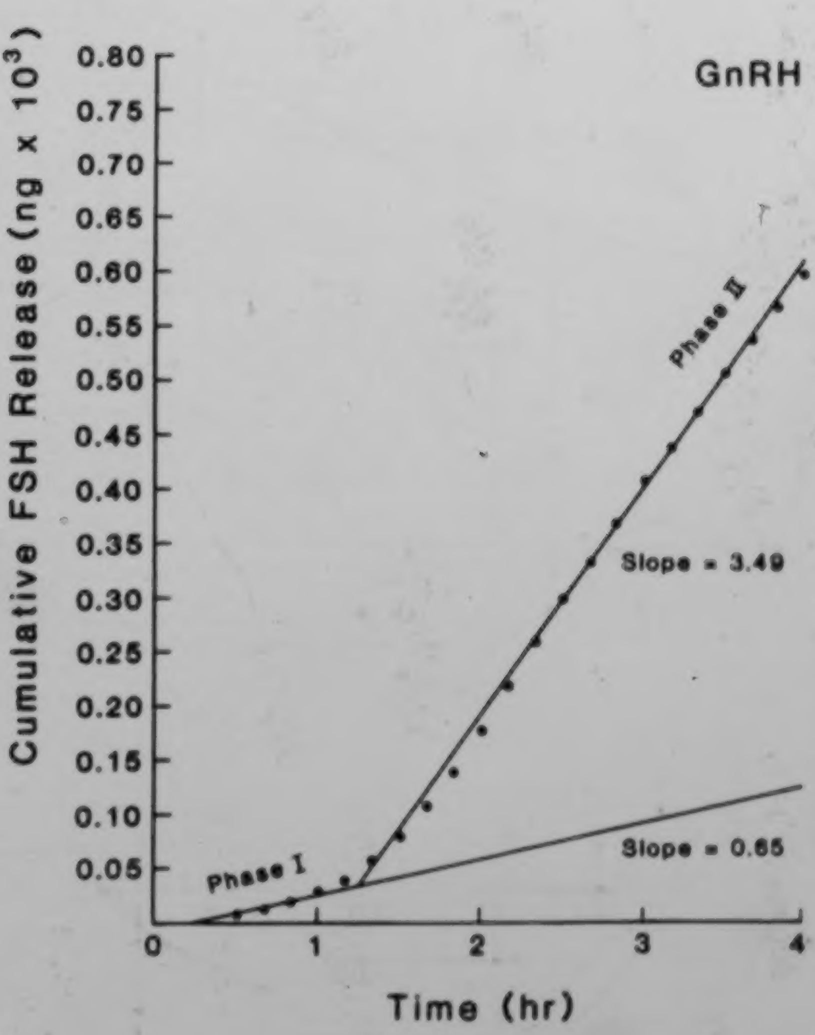

GnRH
Cumulative FSH Release (ng x 10³)
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
1
2
3
4
Time (hr)
Phase I
Phase II
Slope = 3.49
Slope = 0.65

FIG. 6. Secretory responses from pituitaries of diestrous II rats to continuous infusion (4 h) of 1 and 10 μM of ionomycin (indicated by the black bar). Results are the means ± SEM (n=3). For further details, see legend for Fig. 4.

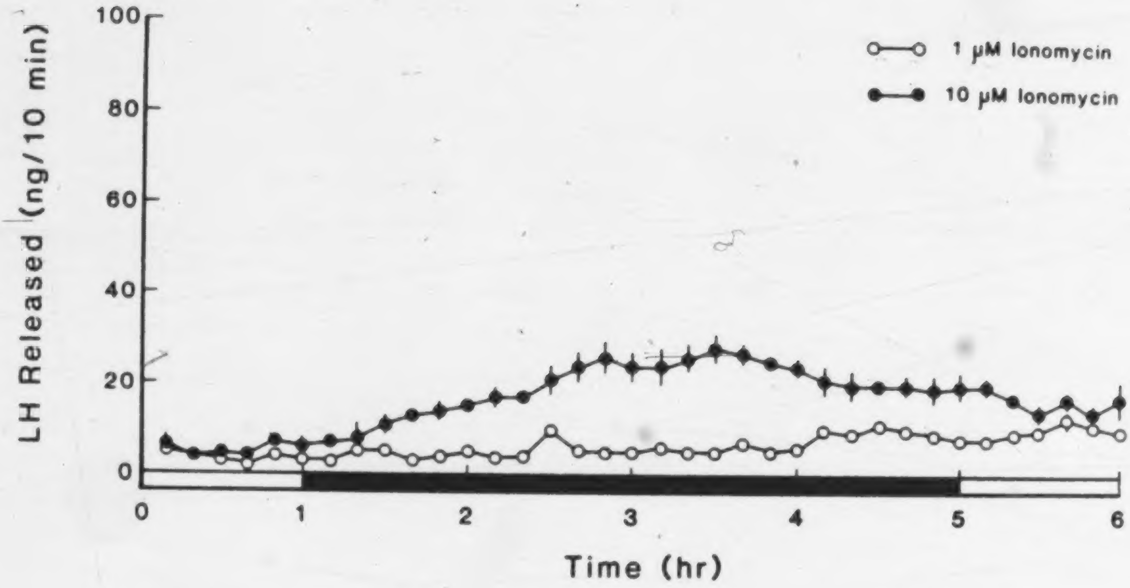
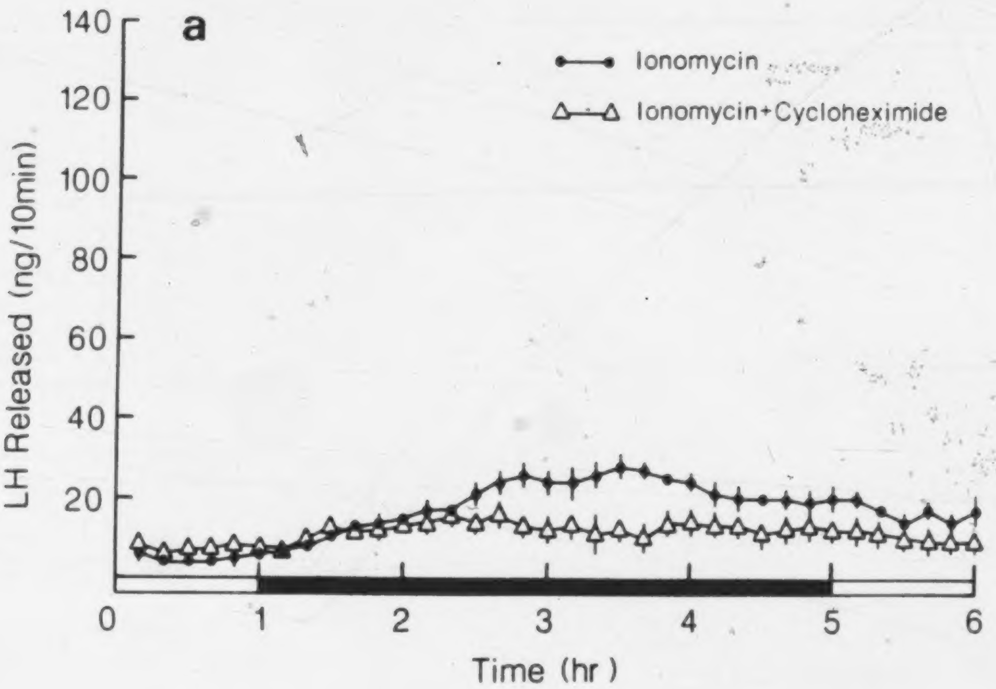
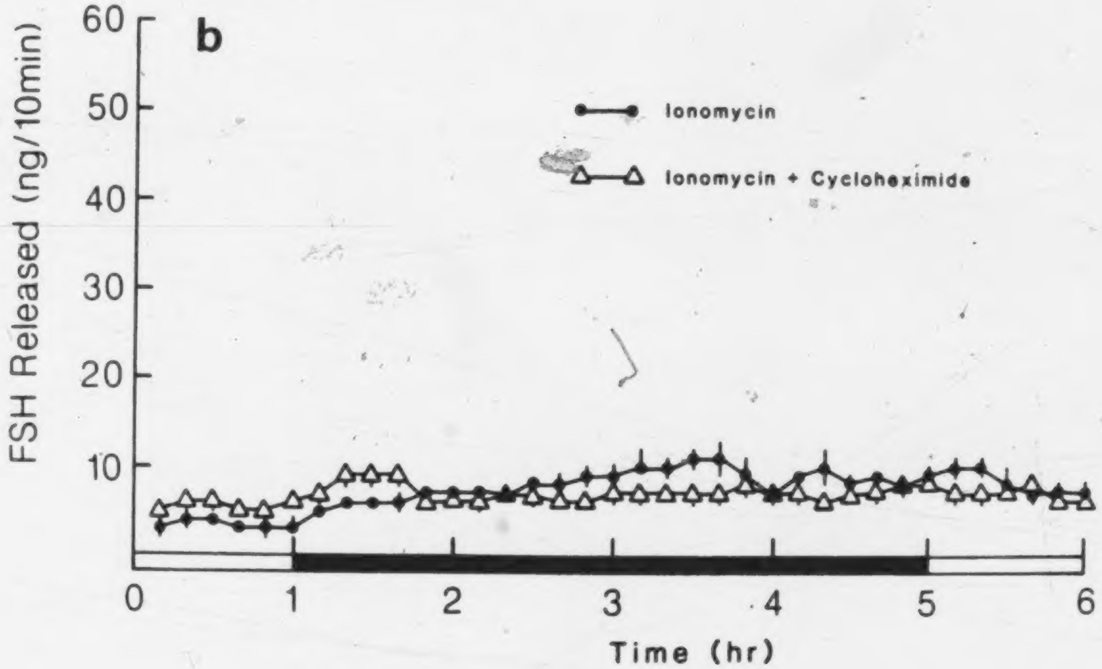

LH Released (ng/10 min)
100
80
60
40
20
0
1 μM Ionomycin
10 μM Ionomycin
0
1
2
3
4
5
6
Time (hr)

FIG.7. Secretory profiles of LH and FSH obtained from pituitaries of diestrous II rats in response to continuous infusion (4 h) of 10 μM of ionomycin (indicated by the black bar) in the absence or presence of 5 μM of cycloheximide (n=3). For further details, see legend for Fig. 4.


a
LH Released (ng/10min)
140
120
100
80
60
40
20
0
1
2
3
4
5
6
Time (hr)
Ionomycin
Ionomycin+Cycloheximide

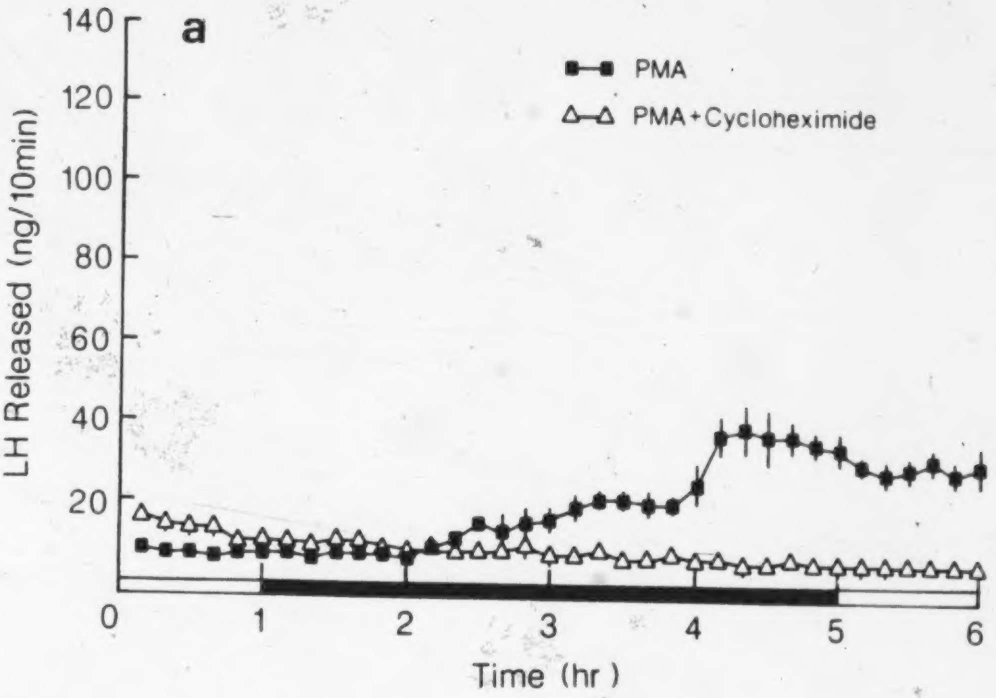

b
FSH Released (ng/10min)
60
50
40
30
20
10
0
1
2
3
4
5
6
Time (hr)
Ionomycin
Ionomycin + Cycloheximide

independent of protein synthesis (Fig.11[a]). The temporal characteristics of the ionomycin response in the presence of cycloheximide suggest that Ca^{++}-mobilization might mediate the GnRH-stimulated phase I release of LH. Surprisingly, cycloheximide almost completely inhibited ionomycin-induced FSH secretion ($p<0.05$) (Fig.7[b] and Fig.11[b]).

Previous reports had indicated that 1 μM was the lowest concentration of PMA which was effective in stimulating appreciable amounts of gonadotropin secretion from perifused female pituitaries (Turgeon and Waring, 1986 and Bourne et al., 1989). Therefore, this concentration was used in all subsequent experiments. In contrast to the results obtained with ionomycin, the secretion of LH induced by 1 μM PMA was delayed, becoming apparent approximately 80 min after its administration, and remained elevated even after PMA infusions were terminated (Fig.8[a]). The secretion of FSH in response to PMA was similar to LH with the exception that the onset of secretion occurred about 2 h after the start of the secretagogue infusion (Fig.8[b]). Moreover, the PMA-induced secretions of LH and FSH were completely inhibited by cycloheximide ([$p<0.05$] for LH and FSH) (Fig.8[a,b] and Fig. 11 [a,b]). The effect of cycloheximide and the temporal characteristics of the secretory profiles obtained in response to PMA are consistent with the suggestion that PMA induces a phase II component of LH and FSH secretion.

Simultaneous infusions of PMA and ionomycin resulted in biphasic responses (Fig.9[a,b]). The responses were characterized by an immediate small increase in gonadotropin secretion, followed about 60 min later by a greatly augmented secretory rate. As was the case with GnRH, cycloheximide completely inhibited the augmented secretion ([$p<0.05$] for LH and FSH) (Fig.9[a,b] and Fig. 11 [a,b]), resulting in a secretory profile that resembled GnRH-stimulated phase I secretion (cf. Fig.4[a,b]). These results indicate that the simultaneous activation of cellular mechanisms by Ca^{++} mobilization and PMA, duplicate the protein synthesis-dependent and -independent characteristics of the biphasic

FIG. 8. Secretory profiles of LH and FSH obtained from pituitaries of diestrous II rats in response to continuous infusion (4 h) of 1 μM of PMA (indicated by the black bar) in the absence or presence of 5 μM of cycloheximide (n=3). For further details, see legend for Fig. 4.


a
PMA
PMA+Cycloheximide
LH Released (ng/10min)
140
120
100
80
60
40
20
0
1
2
3
4
5
6
Time (hr)

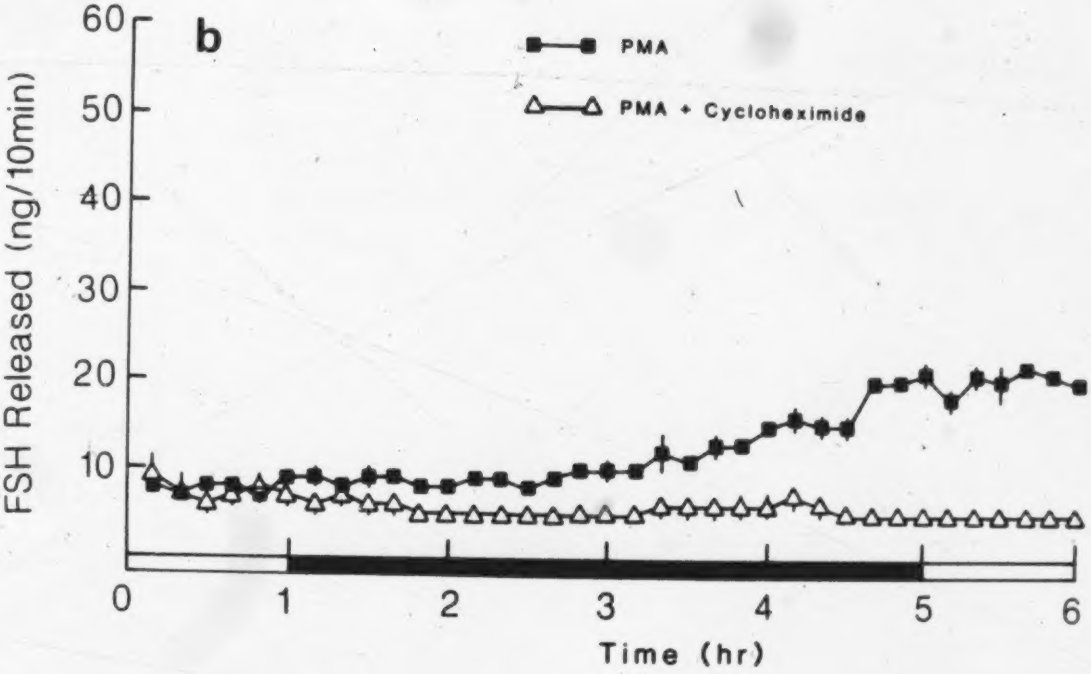
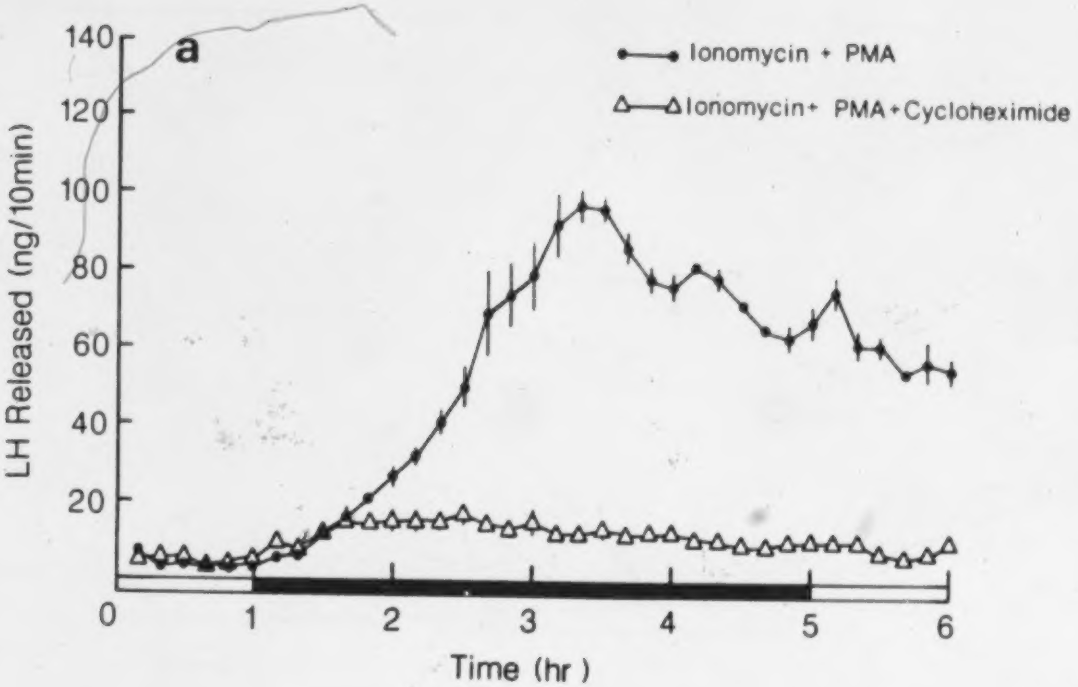
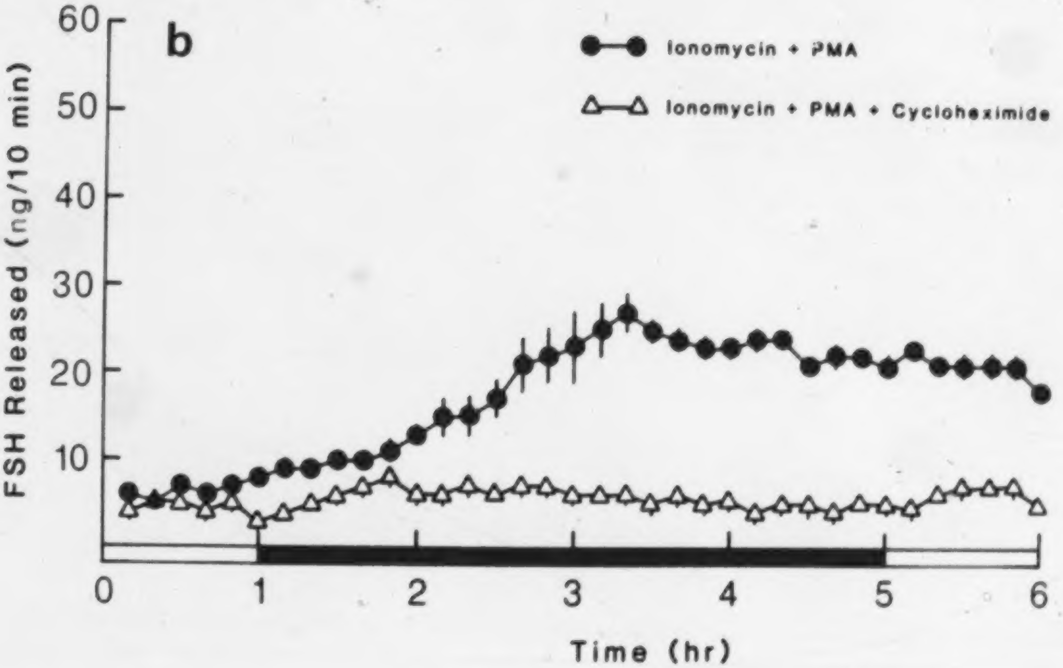

b
PMA
PMA + Cycloheximide
FSH Released (ng/10min)
60
50
40
30
20
10
0
1
2
3
4
5
6
Time (hr)

FIG 9. Secretory profiles of LH and FSH obtained from pituitaries of diestrous II rats in response to continuous infusions (4 h) of ionomycin (10 μM) + PMA (1 μM) simultaneously (indicated by the black bar) in the absence or presence of 5 μM of cycloheximide (n=3). For further details, see legend for Fig. 4.


a
LH Released (ng/10min)
140
120
100
80
60
40
20
0
1
2
3
4
5
6
Time (hr)
Ionomycin + PMA
Ionomycin+ PMA+Cycloheximide


b
FSH Released (ng/10 min)
60
50
40
30
20
10
0
1
2
3
4
5
6
Time (hr)
Ionomycin + PMA
Ionomycin + PMA + Cycloheximide

secretion of LH and FSH. Interestingly, the total amounts of LH and FSH released in response to simultaneous infusions of ionomycin and PMA in the presence of cycloheximide are similar to the amounts obtained with GnRH infusion in the presence of cycloheximide (Fig.11[a,b]).

The suggestion that simultaneous infusions of ionomycin and PMA result in a biphasic secretion was strengthened by plotting the cumulated amounts of LH and FSH (secreted in response to simultaneous infusions of ionomycin and PMA) as a function of time. The results are shown in Fig.10[a,b]. There are two distinct slopes ([$p<0.001$] for LH and FSH) for the "mean" rates of release, with the second phase secretion demonstrating an increased (augmented) secretion rate.

The total amounts of LH and FSH secreted in response to the simultaneous administration of ionomycin and PMA were greater than the algebraic sum of the hormones released in response to the individual secretagogues ([$p<0.05$] for LH and FSH) (Fig.11[a,b]), illustrating the occurrence of synergistic interactions between Ca^{++} mobilization- and PMA-activated mechanisms. Moreover, the secretion arising from these synergistic interactions was dependent on de novo protein synthesis (Fig. 9[a,b] and Fig.11[a,b]), suggesting that these responses represented phase II secretory mechanisms.

FIG. 10a. Cumulated amounts of LH released from pituitaries of diestrous II rats in response to continuous infusions (4 h) of ionomycin (10 µM) + PMA (1 µM) simultaneously. The "mean" secretory rate for phase I is 17.4 ng/10 min, while the corresponding value for phase II is 75.1 ng/10 min. The correlation coefficients for the lines fitted to the data for phase I and phase II are 0.9903 and 0.9981, respectively. For further details, see legend for Fig. 5a.

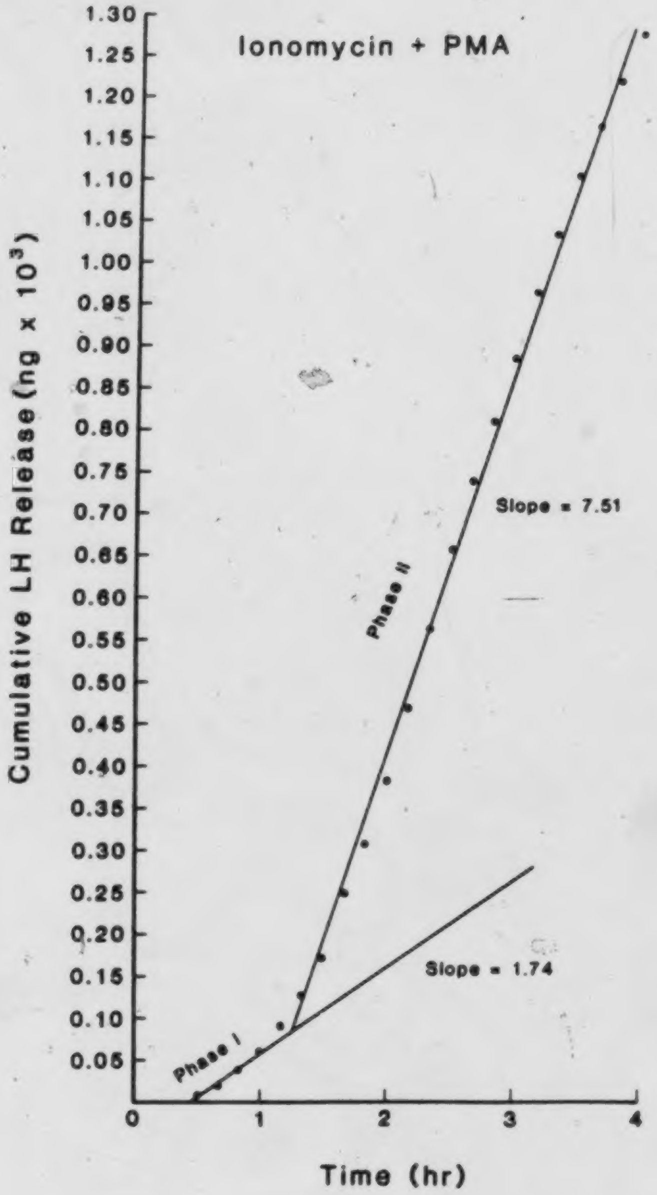

Ionomycin + PMA
Cumulative LH Release (ng x 10³)
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
1
2
3
4
Time (hr)
Phase I
Phase II
Slope = 7.51
Slope = 1.74

FIG. 10b. Cumulated amounts of FSH released from pituitaries of diestrous II rats in response to continuous infusions (4 h) of ionomycin (10 μM) + PMA (1 μM) simultaneously. The "mean" secretory rate for phase I is 3.8 ng/10 min, while the corresponding value for phase II is 14.0 ng/10 min. The correlation coefficients for the lines fitted to the data for phase I and phase II are 0.9947 and 0.9985, respectively. For further details, see legend for Fig. 5a.

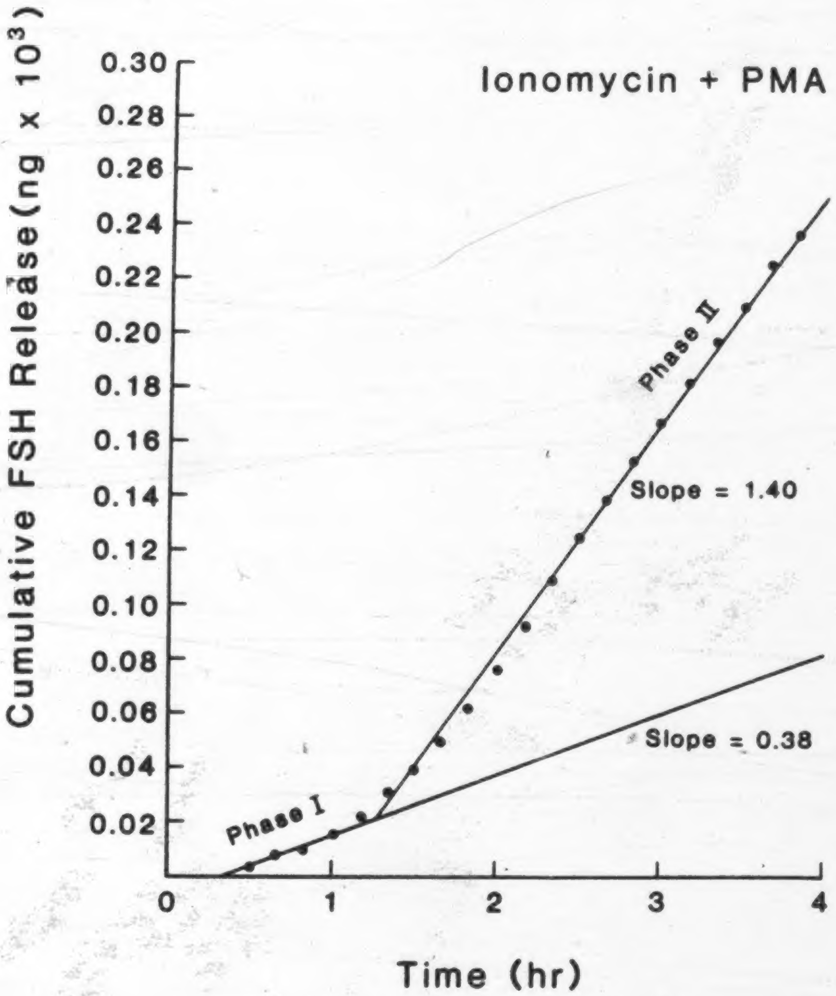

Ionomycin + PMA
Cumulative FSH Release (ng x 10^3)
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
1
2
3
4
Time (hr)
Phase I
Phase II
Slope = 1.40
Slope = 0.38

FIG. 11a. Total amounts of LH released (above basal levels) from pituitaries of diestrous II rats in response to GnRH (1 nM), ionomycin (Iono, 10 μM), PMA (1 μM) and ionomycin + PMA in the absence (represented by blank bars) or presence (hatched bars) of cycloheximide (C, 5 μM). The results are the means ± SEM (n=3). * is indicative of the absence of a response which was greater than basal secretion. The amounts of the hormone were calculated as described in the "Materials and Methods" section. Data for statistical analysis appear in the text.

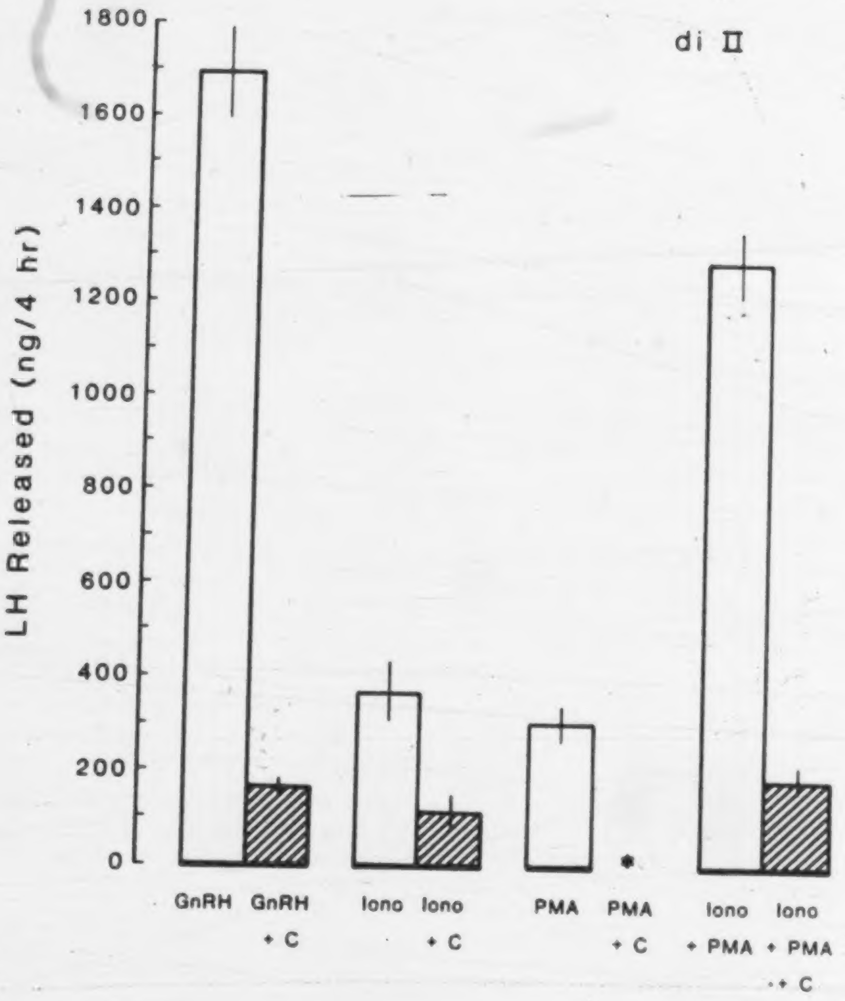
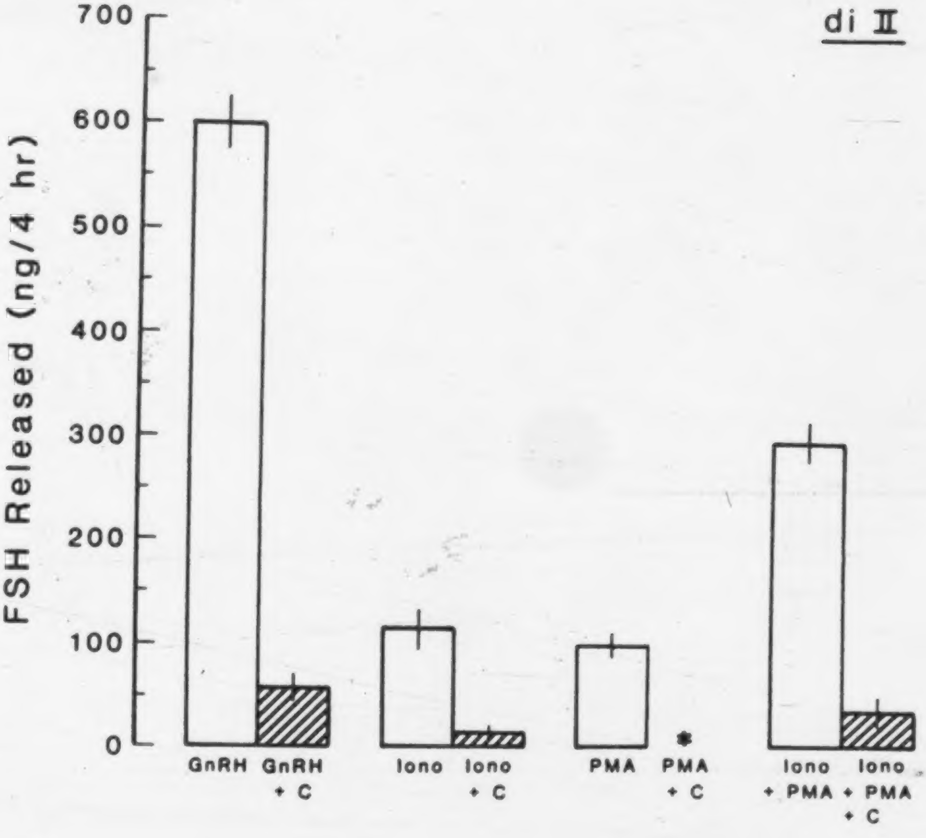

di II
LH Released (ng/4 hr)
1800
1600
1400
1200
1000
800
600
400
200
0
GnRH
GnRH + C
Iono
Iono + C
PMA
PMA + C
Iono + PMA
Iono + PMA + C

FIG. 11b. Total amounts of FSH released (above basal levels) from pituitaries of diestrous II rats in response to GnRH (1 nM), ionomycin (Iono, 10 μM), PMA (1 μM) and ionomycin + PMA in the absence (blank bars) or presence (hatched bars) of cycloheximide (C, 5 μM). Statistical data are included in the text. For further details, see legend for Fig. 11a.


di II
FSH Released (ng/4 hr)
700
600
500
400
300
200
100
0
GnRH
GnRH + C
Iono
Iono + C
PMA
PMA + C
Iono + PMA
Iono + PMA + C
*

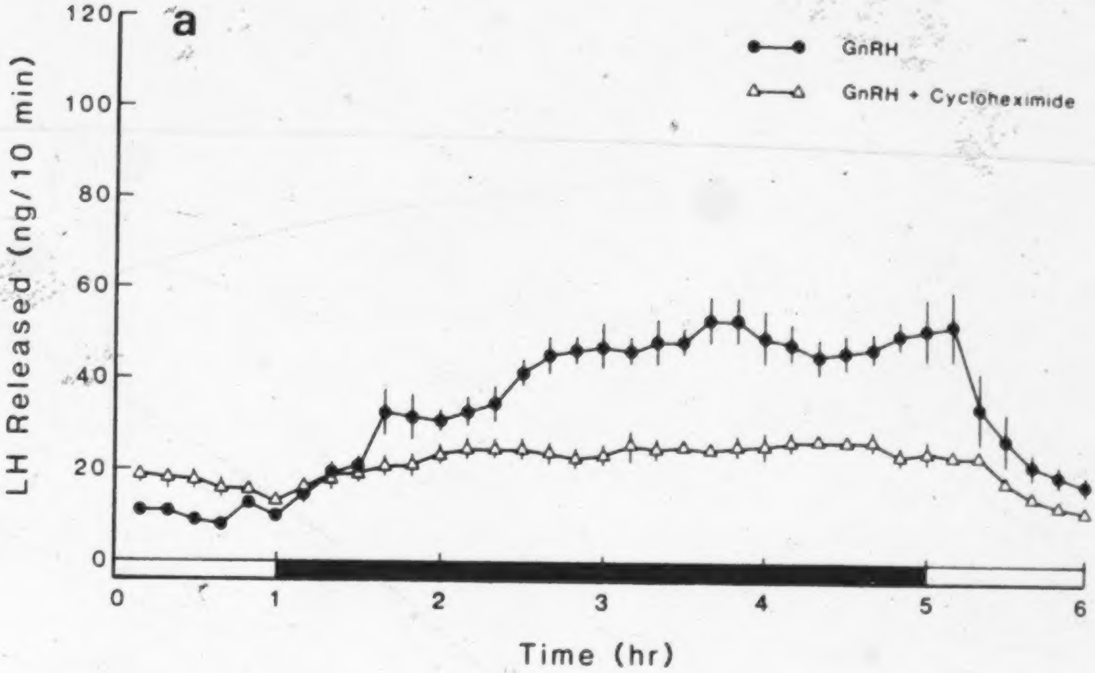
B. Effects of Ionomycin and PMA on Gonadotropin Secretion from Pituitaries of OVX Rats

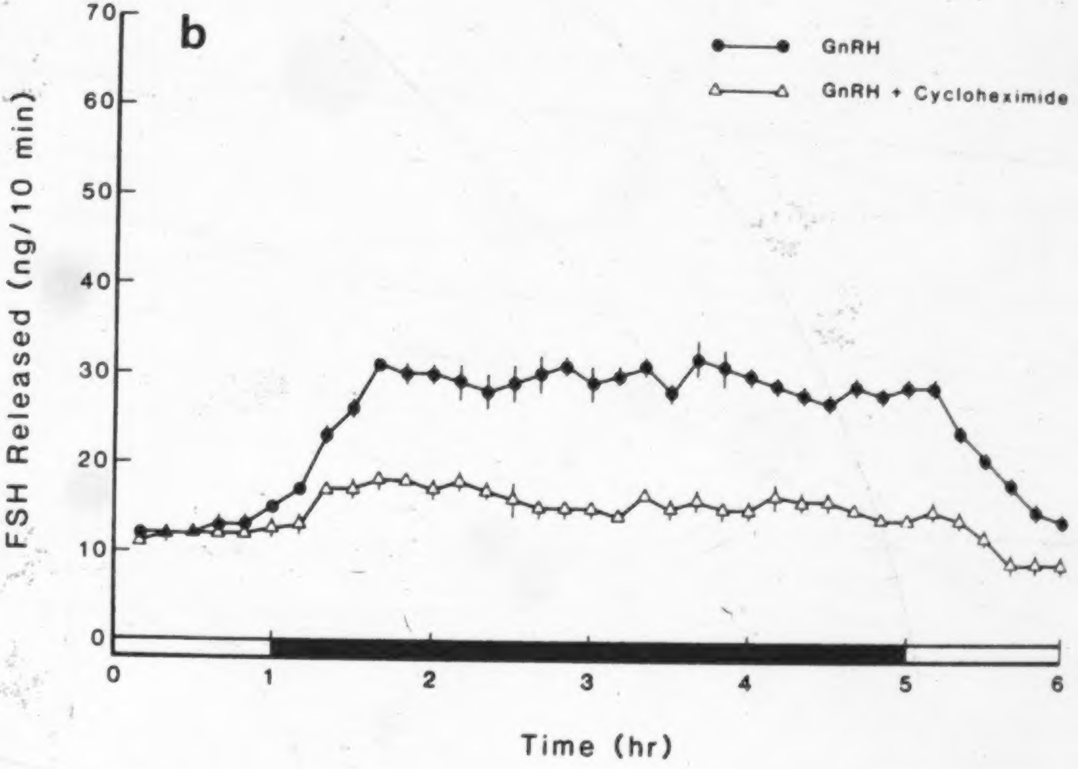
In the previous study, the roles/interactions of the branches of the Ca^{++} messenger system were delineated with respect to the various components of the biphasic secretion of gonadotropins from pituitaries of diestrous II rats. However, earlier studies had demonstrated that ovariectomy results in a marked attenuation of the phase II secretion of the biphasic response of gonadotropins (Baldwin et al., 1983). Ovariectomy also resulted in a complete inhibition of the phase II, extracellular Ca^{++}-independent component of gonadotropin secretion (Bourne et al., 1988). Taken together, these observations suggest that ovariectomy results in changes in the roles/interactions of the mediators of GnRH actions in gonadotropes. Consequently, the present study was undertaken to ascertain whether ovariectomy results in changes in the roles/interactions of the different branches of the Ca^{++} messenger system.

Initial experiments were undertaken to characterize the secretory profiles of LH and FSH obtained from pituitaries of OVX rats in response to continuous infusions of GnRH. Infusions of 1 nM of GnRH for 4 h resulted in a slow, steady increase in LH secretion for 1.5 h, at which time the secretory rate stabilized until the infusion of the decapeptide was terminated (Fig.12[a]). The secretory profile of FSH differed from that of LH in that the FSH response was characterized by a faster increase which stabilized after 40 min, and remained at that level for the duration of GnRH administration (Fig.12[b]). Cycloheximide inhibited a major component of the GnRH-stimulated LH and FSH responses (cf. Fig.12[a,b]), indicating the presence of a small, protein synthesis-independent component of gonadotropin secretion from pituitaries of OVX rats (Fig.18[a,b]).

When the cumulated amount of LH released is plotted as a function of time, the GnRH-stimulated response is seen to be biphasic with a "mean" phase I secretory rate of 20.3 ng/10 min, and a significantly different "mean" secretory rate of 38.8 ng/10 min for

FIG. 12. Secretory profiles of LH and FSH obtained from pituitaries of OVX rats in response to continuous infusion (4 h) of 1 nM of GnRH (indicated by the black bar) in the absence or presence of 5 μM of cycloheximide (n=3). For further details, see legend for Fig. 4.


a
GnRH
GnRH + Cycloheximide
LH Released (ng/10 min)
120
100
80
60
40
20
0
0
1
2
3
4
5
6
Time (hr)


b
GnRH
GnRH + Cycloheximide
FSH Released (ng/10 min)
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
Time (hr)

phase II ($p<0.001$) (Fig.13[a]). It should be noted that the phase II secretory rate from pituitaries of OVX rats is greatly attenuated when compared to pituitaries of diestrous II rats ("mean" secretory rate 100.7 ng/10 min). Ovariectomy resulted in an apparent uniphasic FSH secretory response with a "mean" secretory rate of 16.9 ng/10 min (Fig.13[b]).

The secretory profile of LH obtained in response to constant infusions of ionomycin is shown in Fig.14[a]. LH secretion increased steadily during the first hour of ionomycin infusion and remained at a steady rate for the remainder of the experimental period (Fig.14[a]). Cycloheximide did not affect the ionomycin-induced LH response (Fig.14[a] and cf. Fig.18[a]).

Ionomycin also stimulated the secretion of FSH which fluctuated throughout the duration of the secretagogue administration. Interestingly, cycloheximide, which did not affect ionomycin-stimulated LH secretion, inhibited a significant portion of the ionomycin-induced FSH secretion ($p<0.05$) (Fig.14[b] and Fig.18[b]).

In contrast to the results obtained with ionomycin, PMA was totally ineffective in stimulating LH or FSH secretion from pituitaries of OVX rats (Fig.15[a,b]). It should be emphasized that the inability of PMA to stimulate gonadotropin release in the present study is distinctly different from its efficacy when administered to pituitaries from diestrous II rats.

The secretory profiles of gonadotropins obtained from pituitaries of OVX rats in response to simultaneous infusions of ionomycin and PMA are shown in Fig. 16[a,b]. It is readily apparent that PMA interacted with ionomycin to augment the secretion of LH and FSH, in spite of the fact that the phorbol ester alone did not affect gonadotropin secretion (cf. Fig.15[a,b] and Fig.16[a,b]). Cycloheximide inhibited the augmented secretory responses ([$p<0.05$] for LH and FSH) (Fig.16[a,b] and Fig.18[a,b]), resulting in secretory profiles that resembled the secretory patterns obtained during infusions of ionomycin alone.

FIG. 13a. Cumulated amounts of LH released from pituitaries of OVX rats in response to continuous infusion (4 h) of 1 nM of GnRH. The "mean" secretory rate for phase I is 20.3 ng/10 min, while the corresponding value for phase II is 38.8 ng/10 min. The correlation coefficients for the lines fitted to the data for phase I and phase II are 0.9963 and 0.9997, respectively. For further details, see legend for Fig. 5a.

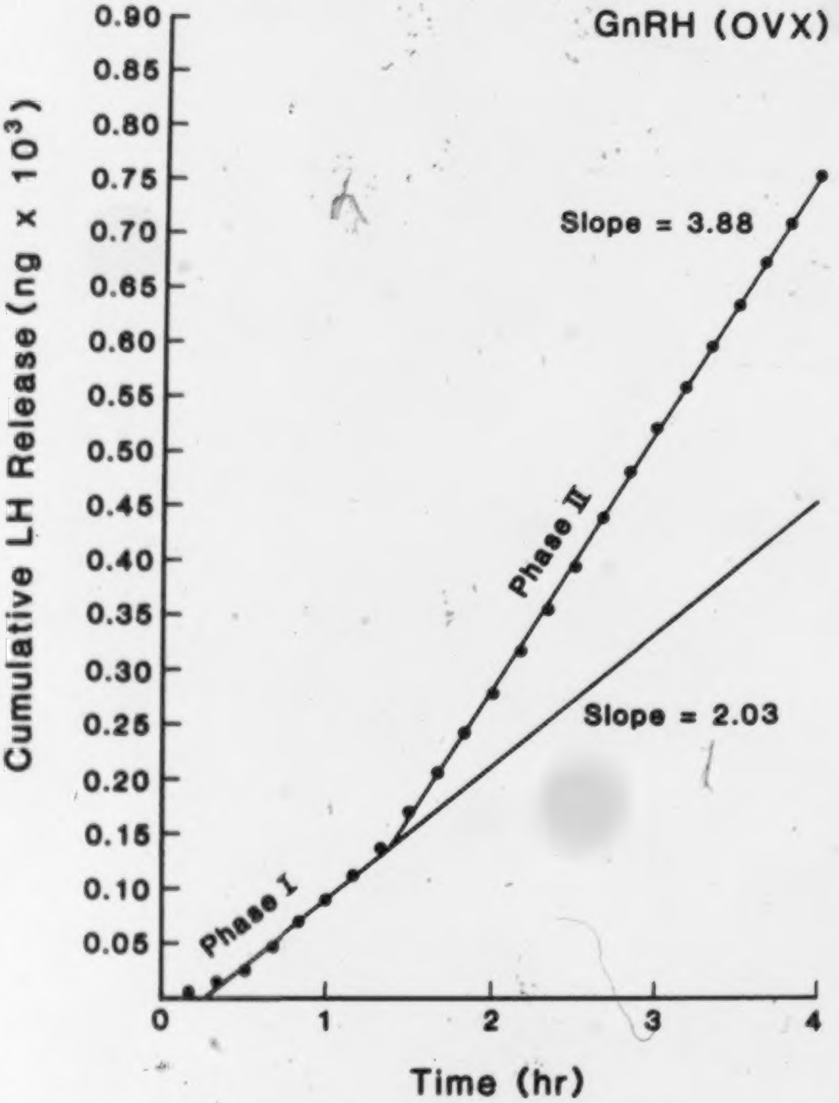

GnRH (OVX)
Cumulative LH Release (ng x 10^3)
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
1
2
3
4
Time (hr)
Slope = 3.88
Slope = 2.03
Phase I
Phase II

FIG. 13b. Cumulated amounts of FSH released from pituitaries of OVX rats in response to continuous infusion (4 h) of 1 nM of GnRH. The "mean" secretory rate is 16.9 ng/10 min. The correlation coefficient is 0.9979. For further details, see legend for Fig. 5a.

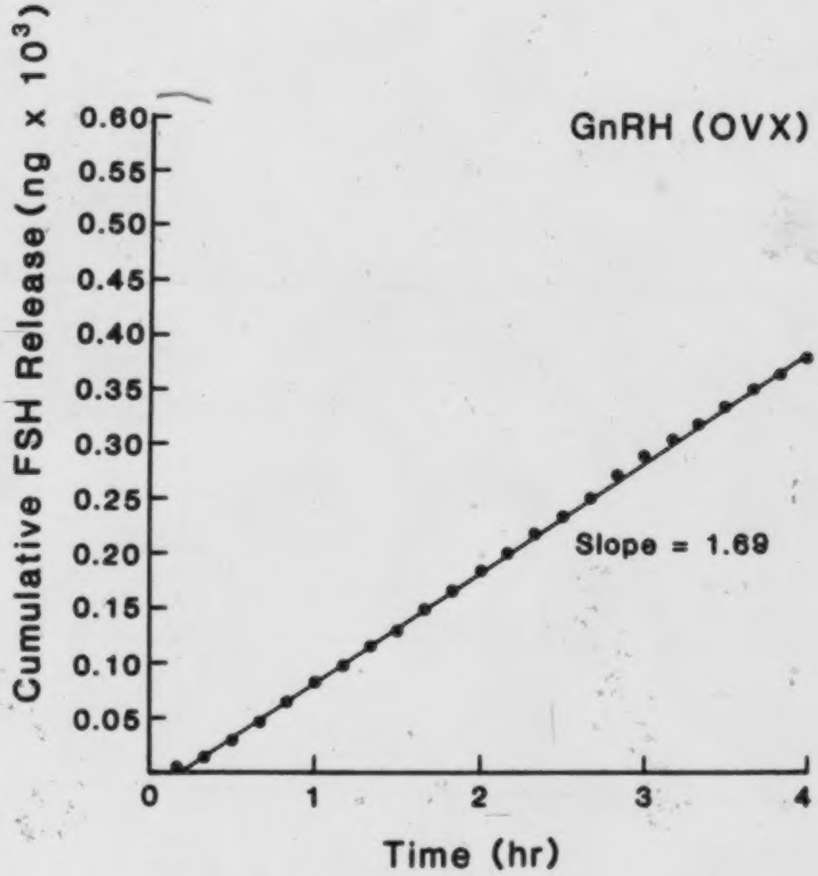
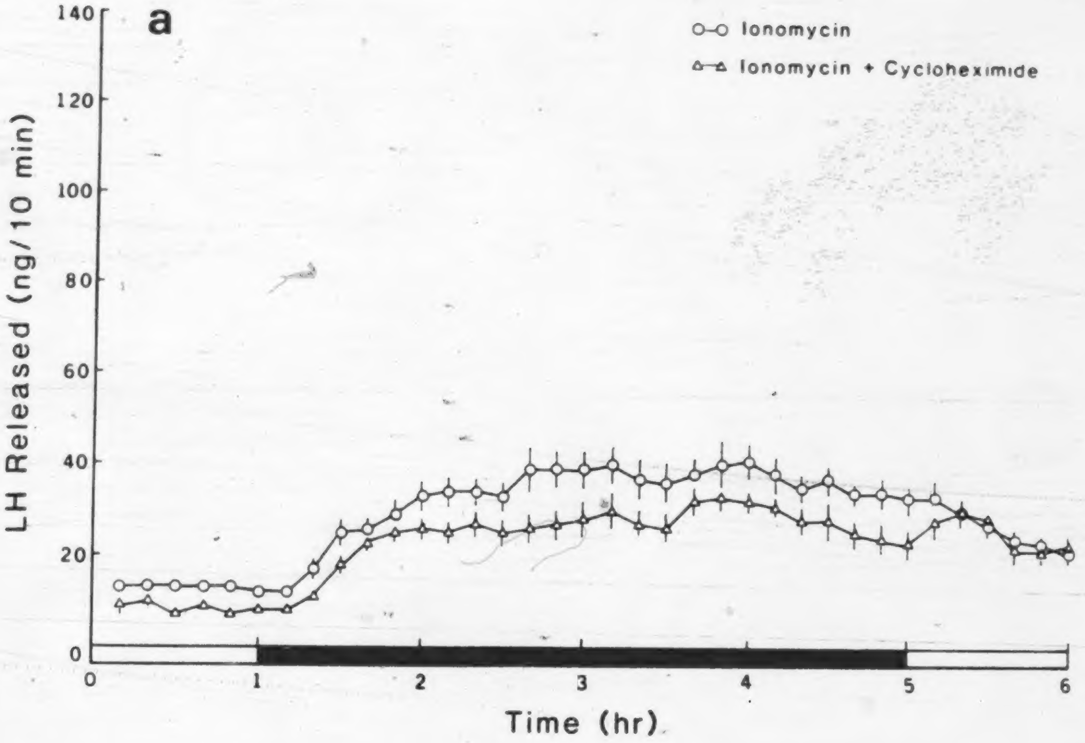

Cumulative FSH Release (ng x 10^3)
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
1
2
3
4
Time (hr)
GnRH (OVX)
Slope = 1.69

FIG. 14. Secretory profiles of LH and FSH obtained from pituitaries of OVX rats in response to continuous infusion (4 h) of 10 μM of ionomycin (indicated by the black bar) in the absence or presence of 5 μM of cycloheximide (n=3). For further details, see legend for Fig. 4.


a
Ionomycin
Ionomycin + Cycloheximide
LH Released (ng/10 min)
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
Time (hr)

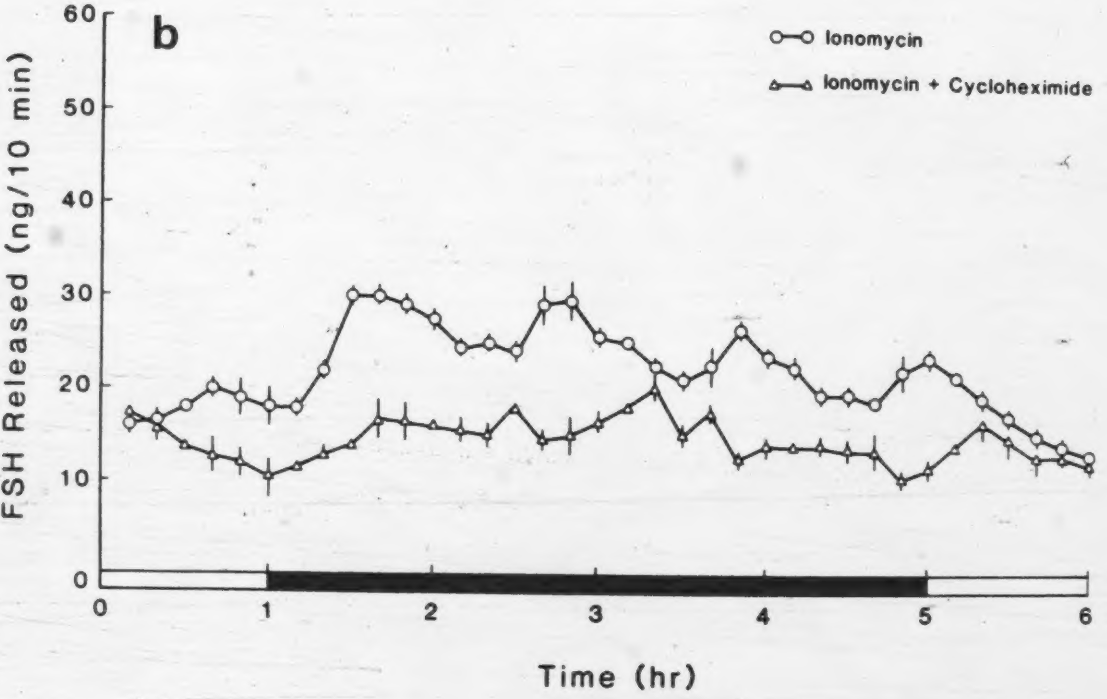
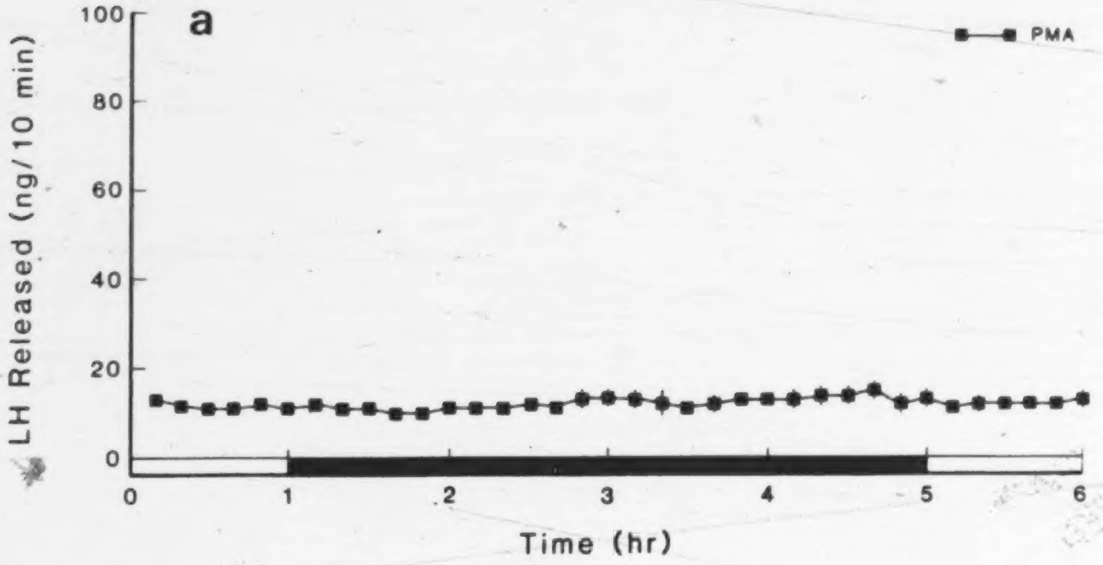

b
Ionomycin
Ionomycin + Cycloheximide
FSH Released (ng/10 min)
60
50
40
30
20
10
0
0
1
2
3
4
5
6
Time (hr)

FIG. 15. Secretory profiles of LH and FSH obtained from pituitaries of OVX rats in response to continuous infusion (4 h) of 1 μM of PMA (indicated by the black bar) (n=3). For further details, see legend for Fig. 4.


a
PMA
LH Released (ng/10 min)
100
80
60
40
20
0
0
1
2
3
4
5
6
Time (hr)

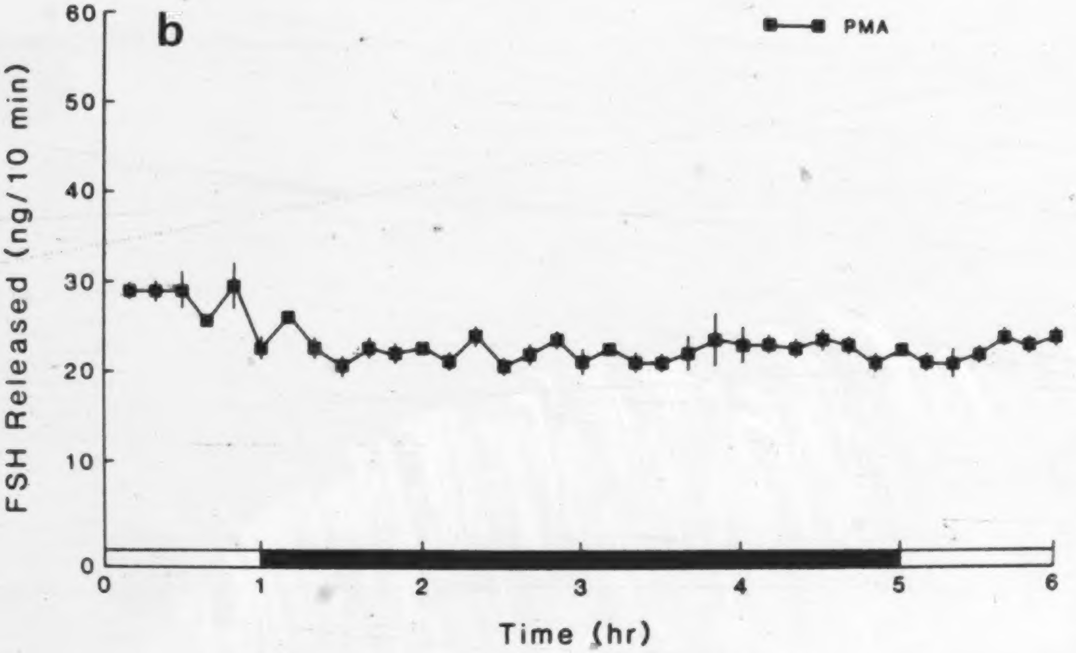
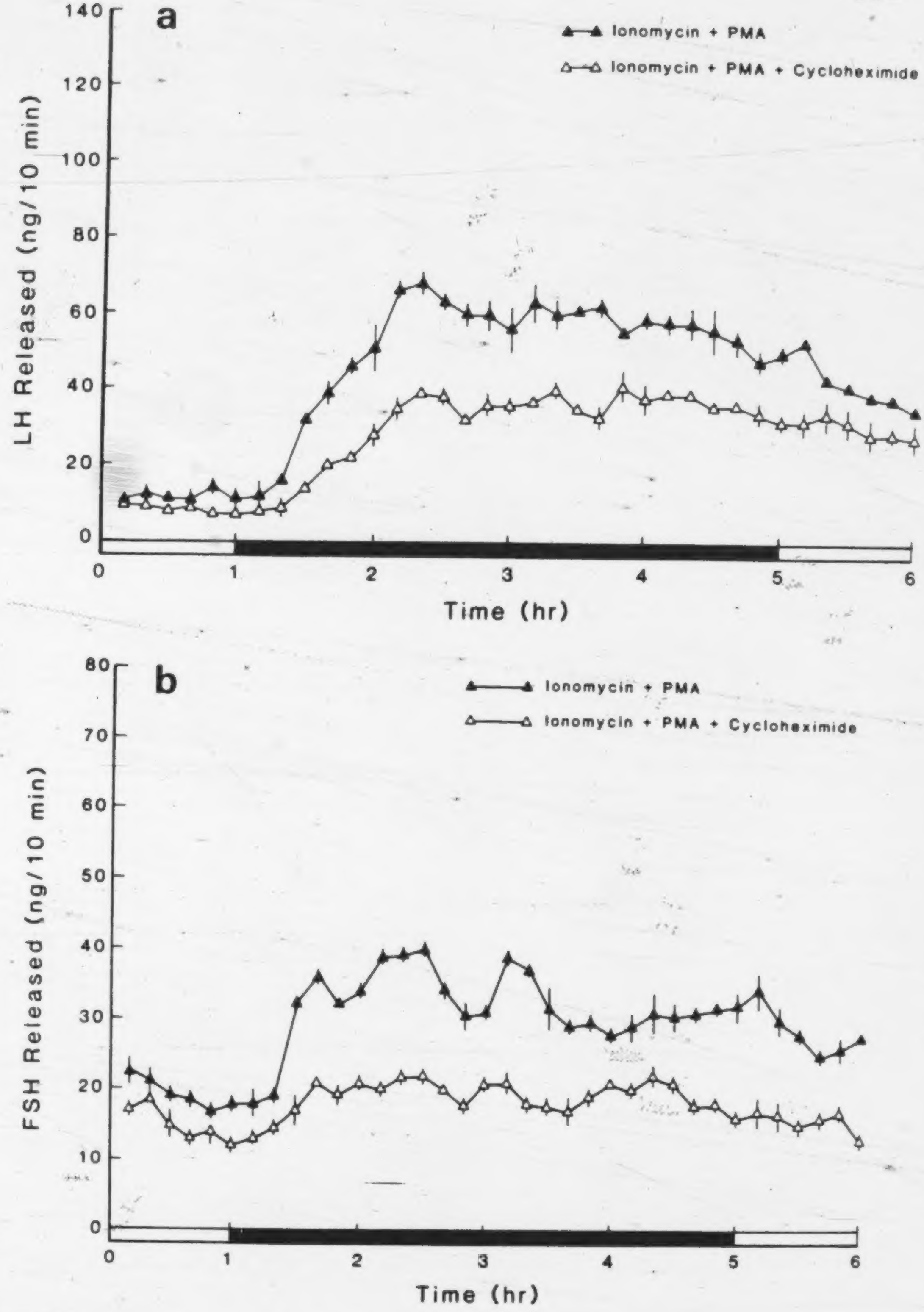

b
PMA
FSH Released (ng/10 min)
60
50
40
30
20
10
0
0
1
2
3
4
5
6
Time (hr)

FIG. 16. Secretory profiles of LH and FSH obtained from pituitaries of OVX rats in response to continuous infusions (4 h) of ionomycin (10 μM) + PMA (1 μM) in the absence or presence of 5 μM of cycloheximide (n=3). For further details, see legend for Fig. 4.


a
Ionomycin + PMA
Ionomycin + PMA + Cycloheximide
LH Released (ng/10 min)
0
20
40
60
80
100
120
140
0
1
2
3
4
5
6
Time (hr)
b
Ionomycin + PMA
Ionomycin + PMA + Cycloheximide
FSH Released (ng/10 min)
0
10
20
30
40
50
60
70
80
0
1
2
3
4
5
6
Time (hr)

The cumulated amounts of LH and FSH secreted in response to simultaneous infusions of PMA and ionomycin plotted as a function of time are shown in Fig.17[a,b]. These agents duplicated (qualitatively) the results of GnRH, by demonstrating a biphasic response for LH. A "mean" phase I secretory rate of 30.6 ng/10 min was followed by a significantly different "mean" secretory rate of 47.1 ng/10 min for phase II ($p<0.001$) (Fig. 17[a]). Ionomycin and PMA also qualitatively duplicated the GnRH-stimulated FSH response, resulting in an apparent uniphasic response consisting of a "mean" secretory rate of 13.8 ng/10 min (Fig. 17 [b]).

The total amounts of LH and FSH secreted in response to the various secretagogues are shown in Fig.18[a,b]. The release obtained from the simultaneous infusions of ionomycin and PMA is significantly greater ([$p<0.05$] for LH and FSH) than the algebraic sum of the responses obtained from ionomycin or PMA alone, indicating synergistic interactions between these two compounds. Interestingly, cycloheximide apparently inhibited only the synergized component of LH and FSH release, since the secretion obtained from the simultaneous administration of ionomycin and PMA in the presence of cycloheximide is similar to that obtained with ionomycin alone.

FIG. 17a. Cumulated amounts of LH released from pituitaries of OVX rats in response to continuous infusions (4 h) of ionomycin (10 μM) + PMA (1 μM) simultaneously. The "mean" secretory rate for phase I is 30.6 ng/10 min, while the corresponding value for phase II is 47.1 ng/10 min. The correlation coefficients for the lines fitted to the data for phase I and phase II are 0.9921 and 0.9994, respectively. For further details, see legend for Fig. 5a.

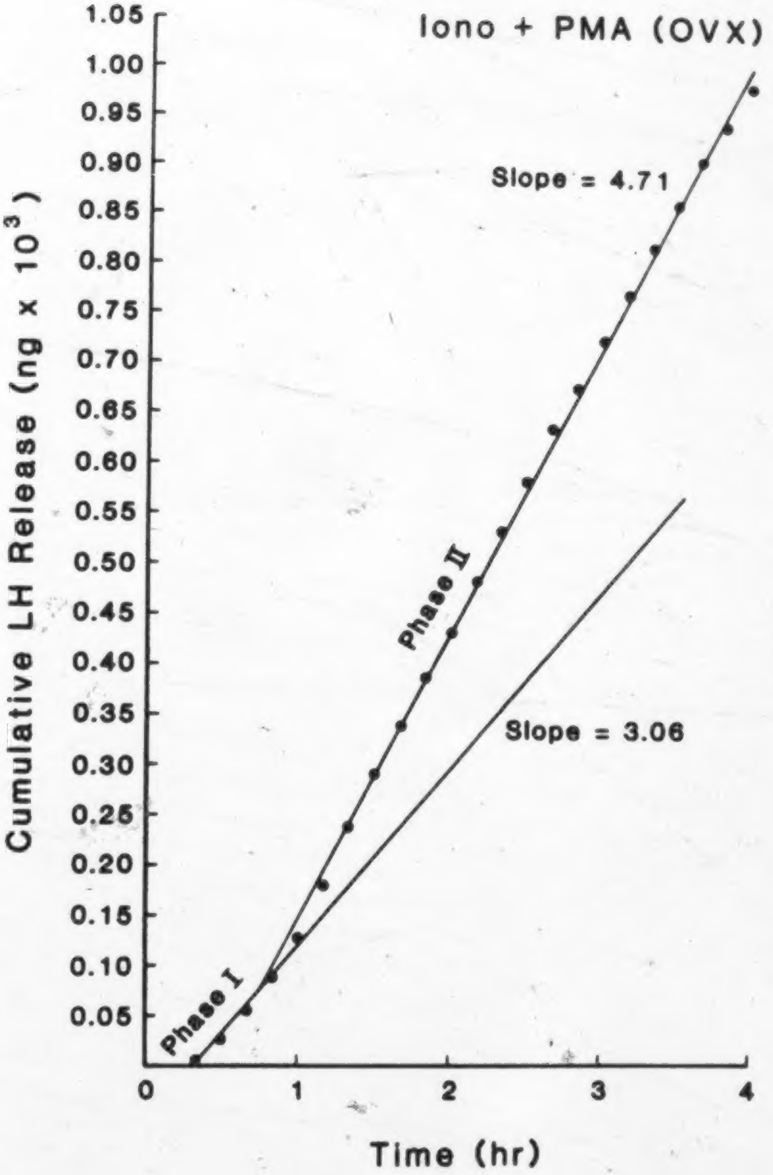

Iono + PMA (OVX)
Cumulative LH Release (ng x 10^3)
1.05
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
1
2
3
4
Time (hr)
Slope = 4.71
Phase II
Slope = 3.06
Phase I

FIG. 17b. Cumulated amounts of FSH released from pituitaries of OVX rats in response to continuous infusions (4 h) of ionomycin (10 µM) + PMA (1 µM) simultaneously. The "mean" secretory rate is 13.8 ng/10 min. The correlation coefficient is 0.9946. For further details, see legend for Fig. 5a.

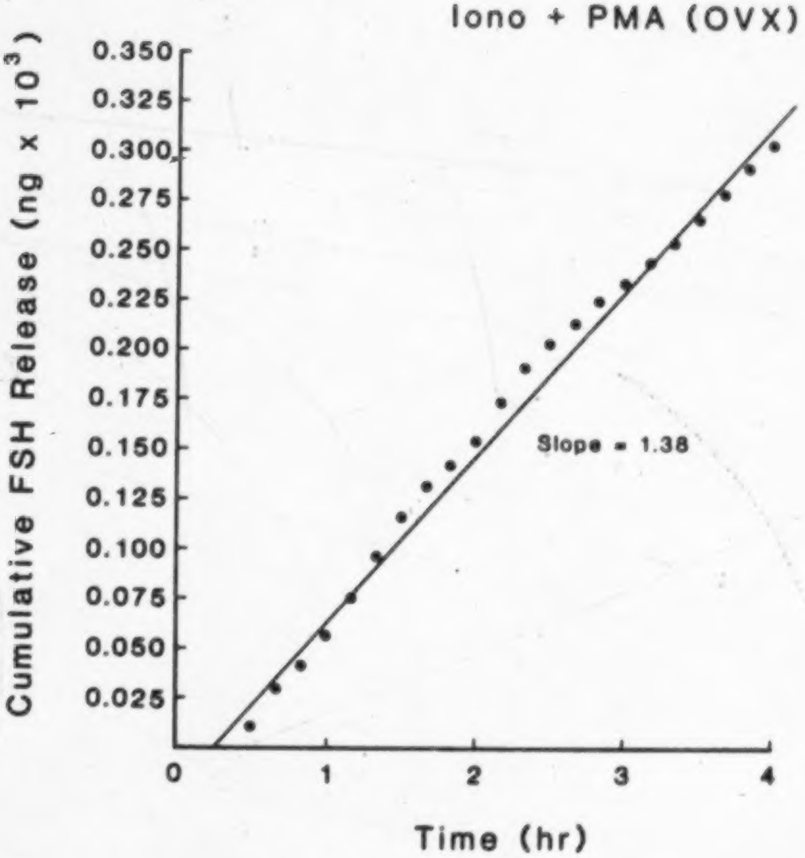

Iono + PMA (OVX)
Cumulative FSH Release (ng x 10^3)
0.350
0.325
0.300
0.275
0.250
0.225
0.200
0.175
0.150
0.125
0.100
0.075
0.050
0.025
0
1
2
3
4
Time (hr)
Slope = 1.38

FIG. 18a. Total amounts of LH released (above basal levels) from pituitaries of OVX rats in response to GnRH (1 nM), ionomycin (Iono, 10 μM), PMA (1 μM) and ionomycin + PMA in the absence (blank bars) or presence (hatched bars) of cycloheximide (C, 5 μM). The results are the means ± SEM (n=3). Data for statistical analysis appear in the text. For further details, see legend for Fig. 11a.

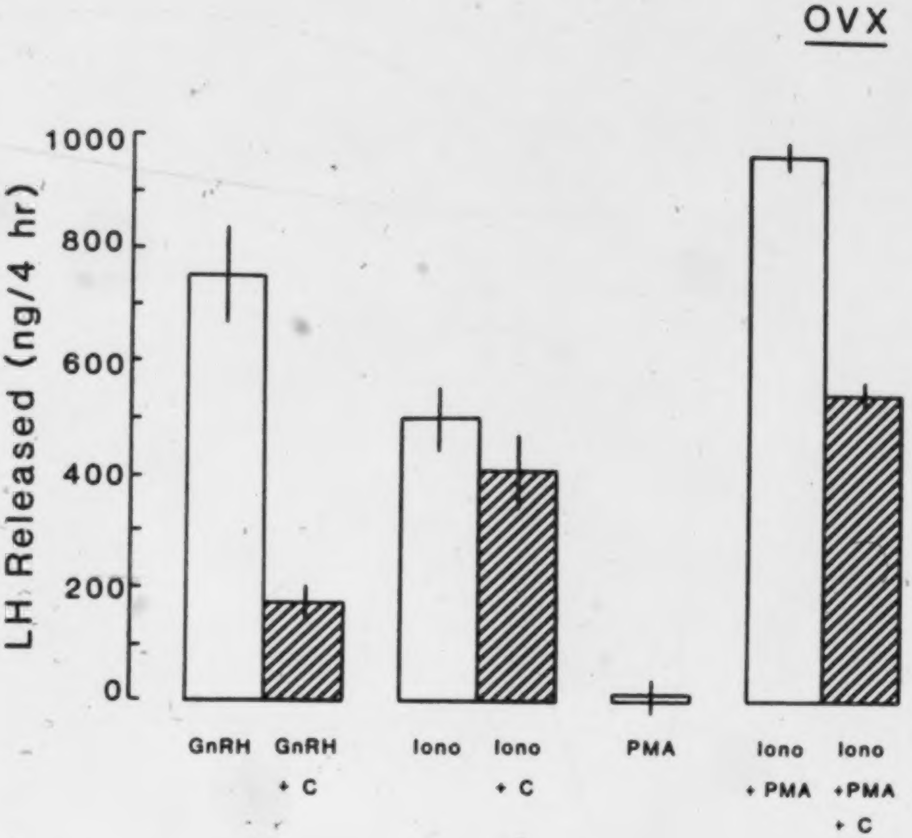
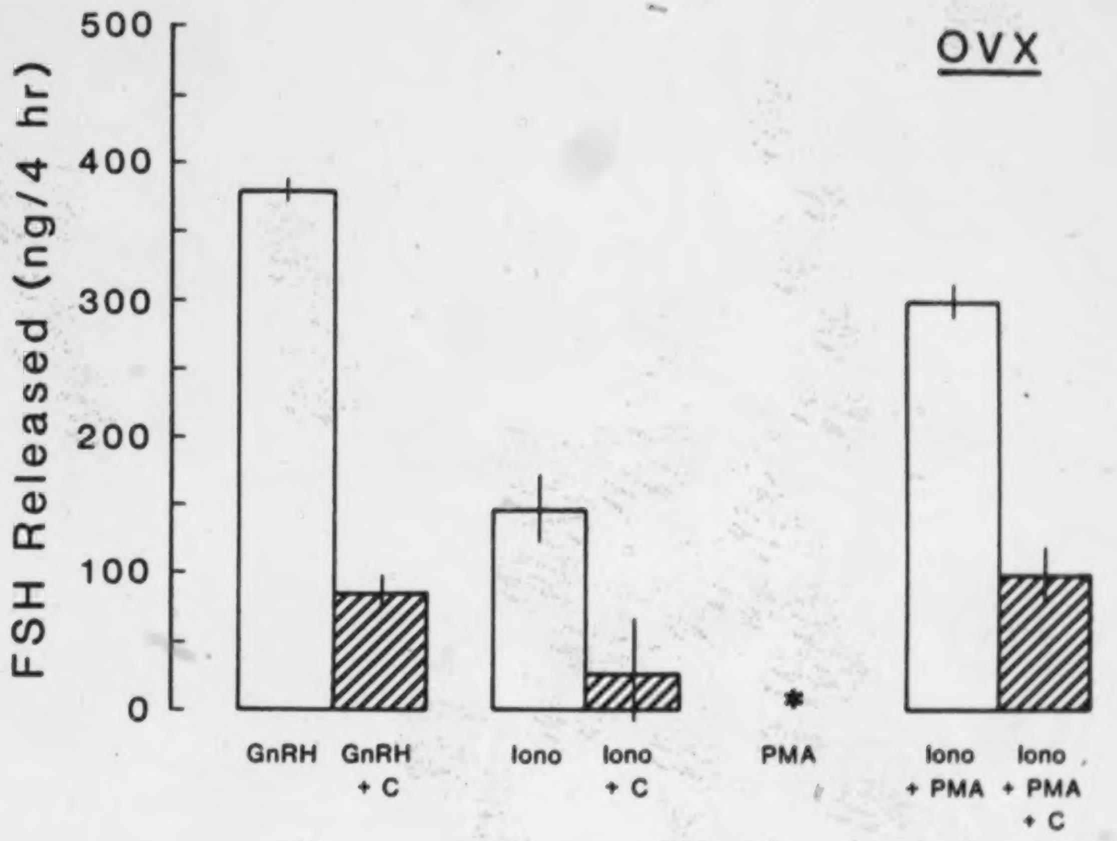

OVX
LH Released (ng/4 hr)
1000
800
600
400
200
0
GnRH
GnRH + C
Iono
Iono + C
PMA
Iono + PMA
Iono +PMA + C

FIG. 18b. Total amounts of FSH released (above basal levels) from pituitaries of OVX rats in response to GnRH (1 nM), ionomycin (Iono, 10 μM), PMA (1 μM) and ionomycin + PMA in the absence (blank bars) or presence (hatched bars) of cycloheximide (C, 5 μM). The results are the means ± SEM (n=3). Statistical data are included in the text. For further details, see legend for Fig. 11a.


OVX
FSH Released (ng/4 hr)
500
400
300
200
100
0
*
GnRH
GnRH + C
Iono
Iono + C
PMA
Iono + PMA
Iono + PMA + C

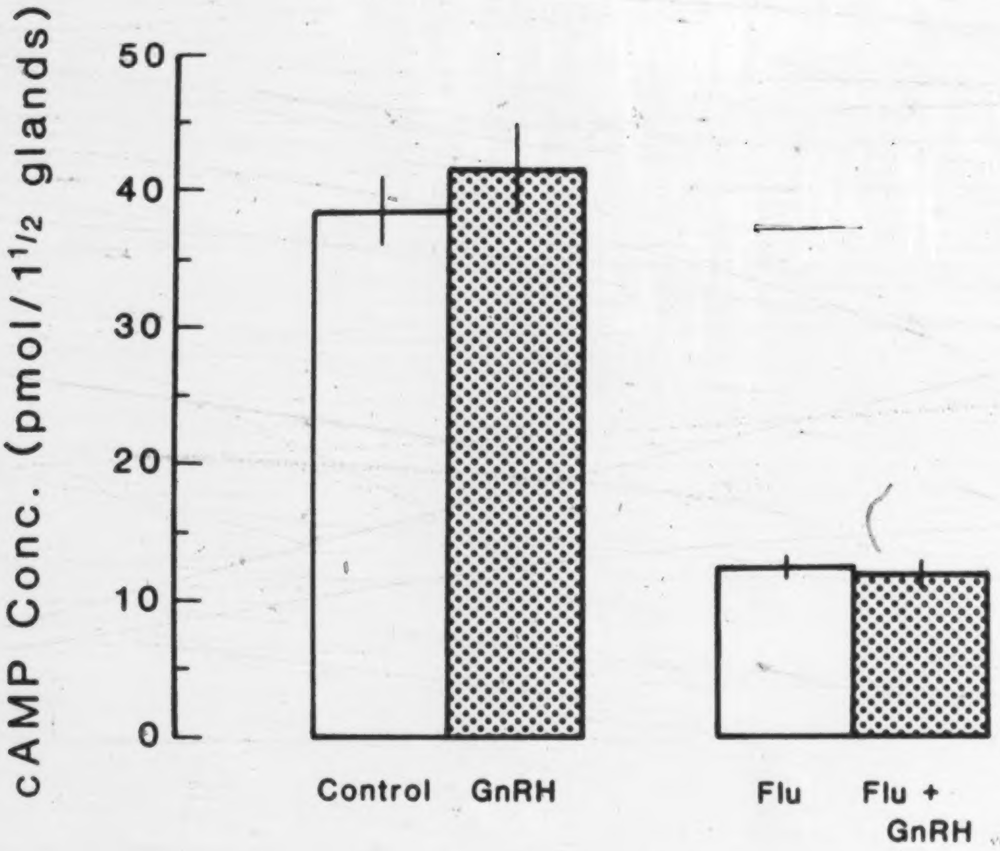
C. Role of Cyclic AMP in GnRH-Stimulated Gonadotropin Secretion from Pituitaries of OVX Rats

The previous study demonstrated that ovariectomy results in the inability of gonadotropes to respond to PMA with increased gonadotropin secretion, i.e. ovariectomy affected some mechanisms of the second phase of secretion of gonadotropins. Interestingly, the phase II gonadotropin secretion from pituitaries of diestrous II rats was shown to be dependent on cAMP (Bourne and Baldwin, 1987[a]). As a result, the following experiments were undertaken to determine whether ovariectomy induces a change in the role(s) of the adenylyl cyclase-cAMP system. The experimental approach was to use a pharmacological agent, Flu, which inhibits GnRH-stimulated cAMP production (Naor et al., 1975[a,b] and Bourne and Baldwin, 1987[a,b]). A previous report had indicated that 0.1 mM Flu was the minimum concentration which inhibited GnRH-stimulated cAMP production in pituitaries obtained from female rats (Bourne and Baldwin, 1987[a]). Consequently, this concentration was used in the subsequent experiments.

Initial static incubatioin experiments were undertaken to demonstrate the effects of Flu on GnRH-stimulated increases in cAMP production in pituitaries from OVX rats. The pituitaries were incubated in the presence of dopamine for reasons discussed in the "Literature Review" section. The concentration of dopamine (10 μM) used in this study was based on the research by Cronin and Thorner (1982). GnRH (75 nM) was ineffective in stimulating an increase in cAMP production in pituitaries incubated in control or (0.1 mM) Flu-containing media (Fig. 19). However, Flu significantly decreased ($p<0.05$) basal cAMP concentrations in the pituitaries (Fig. 19)

Previous studies had demonstrated that in some instances GnRH-stimulated increases in cAMP production could only be demonstrated in the presence of phosphodiesterase inhibitors (Adams et al., 1979[b] and Bourne, 1988). Therefore, the experiments were repeated in the presence of IBMX using the concentration (0.5mM)

FIG. 19. Effect of Flufenamate and GnRH on cAMP in pituitaries of OVX rats. Paired hemipituitaries from OVX rats were incubated (2 h) in the presence or absence of GnRH (75 nM) and/or flufenamate (0.1 mM) in KIRB medium containing dopamine (10 μM). Values represent the means ± SEM (n=4). Data for statistical analysis appear in the text. Abbreviations are defined in the text.


cAMP Conc. (pmol/ 1 1/2 glands)
50
40
30
20
10
0
Control
GnRH
Flu
Flu + GnRH

previously reported (Bourne, 1988). As expected, IBMX increased basal, intracellular cAMP concentrations (Fig. 20). However, GnRH stimulated a significant increase ($p<0.05$) in cAMP production, while Flu inhibited the GnRH-stimulated increase (Fig. 20). Interestingly, Flu also completely inhibited the GnRH-stimulated secretion of LH ($p=0.001$) (Fig. 21), indicating clearly that inhibition of cAMP production was accompanied by an inhibition of GnRH-stimulated LH secretion in this static incubation system.

Perifusion experiments were subsequently undertaken to characterize basal LH and FSH secretion from pituitaries of OVX rats, in addition to determining potential effects of Flu and DBcAMP on gonadotropin secretion. As shown in Fig. 22[a], basal LH secretion declined gradually over the 6 h experimental period. Flu resulted in an apparent elevation of the initial LH secretion which gradually decreased to basal levels after 2.5 h. DBcAMP was ineffective in stimulating LH secretion. Basal FSH secretion which was greater than basal LH secretion, also decreased gradually over the 6 h experimental period (Fig. 22[b]). Flu resulted in a response which was qualitatively similar to that of basal FSH secretion, but was lower in magnitude. DBcAMP did not stimulate FSH secretion.

The effects of Flu on GnRH-stimulated gonadotropin secretion are shown in Fig. 23[a,b]. Flu inhibited a large component of GnRH-stimulated LH and FSH secretion (cf. Fig. 12[a,b]), resulting in secretory profiles characterized by initial responses which gradually decreased to basal levels. Infusions of DBcAMP (in the presence of GnRH and Flu) resulted in an increased gonadotropin secretion, including a potentiation of the initial response (occurring in the first hour) ($p<0.05$) of LH, but not of FSH (Fig. 23[a,b]); Table 1). Moreover, the gonadotropin responses obtained in the presence of DBcAMP appear to be protein synthesis-dependent, in that they were inhibited by cycloheximide ([$p<0.05$] for LH and FSH) (Fig. 23[a,b] and Fig. 25[a,b]). Interestingly, cycloheximide did not affect the initial component (during the first hour) of LH secretion which was potentiated by DBcAMP (Fig. 23[a,b] and Table 1).

FIG. 20. Effect of Flufenamate on GnRH-stimulated cAMP production in pituitaries of OVX rats. Paired hemipituitaries from OVX rats were incubated (2 h) in KIRB medium, containing dopamine (10 µM) and IBMX (0.5 mM) in the presence or absence of GnRH (75 nM) and/or flufenamate (0.1 mM). Values represent the means ± SEM (where n=10 for control + IBMX ± GnRH; and n=4 for IBMX + Flu ± GnRH). Statistical data are included in the text. For further details, see legend for Fig. 19.

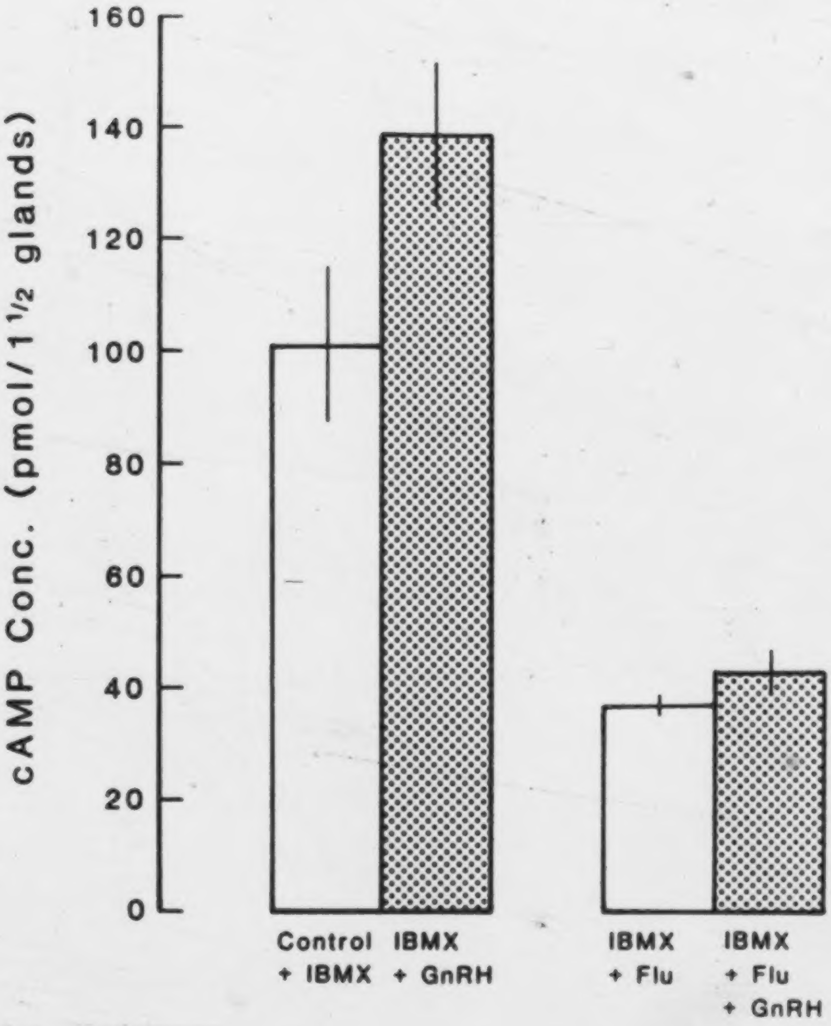

cAMP Conc. (pmol/1 1/2 glands)
160
140
120
100
80
60
40
20
0
Control + IBMX
IBMX + GnRH
IBMX + Flu
IBMX + Flu + GnRH

FIG. 21. LH released from anterior pitutiaries of OVX rats incubated (2 h) in KIRB medium containing dopamine (10 μM) and IBMX (0.5 mM) in the absence or presence of GnRH (75 nM) and/or flufenamate (0.1 mM). Statistical data are included in the text. For further details, see legend for Fig. 20.

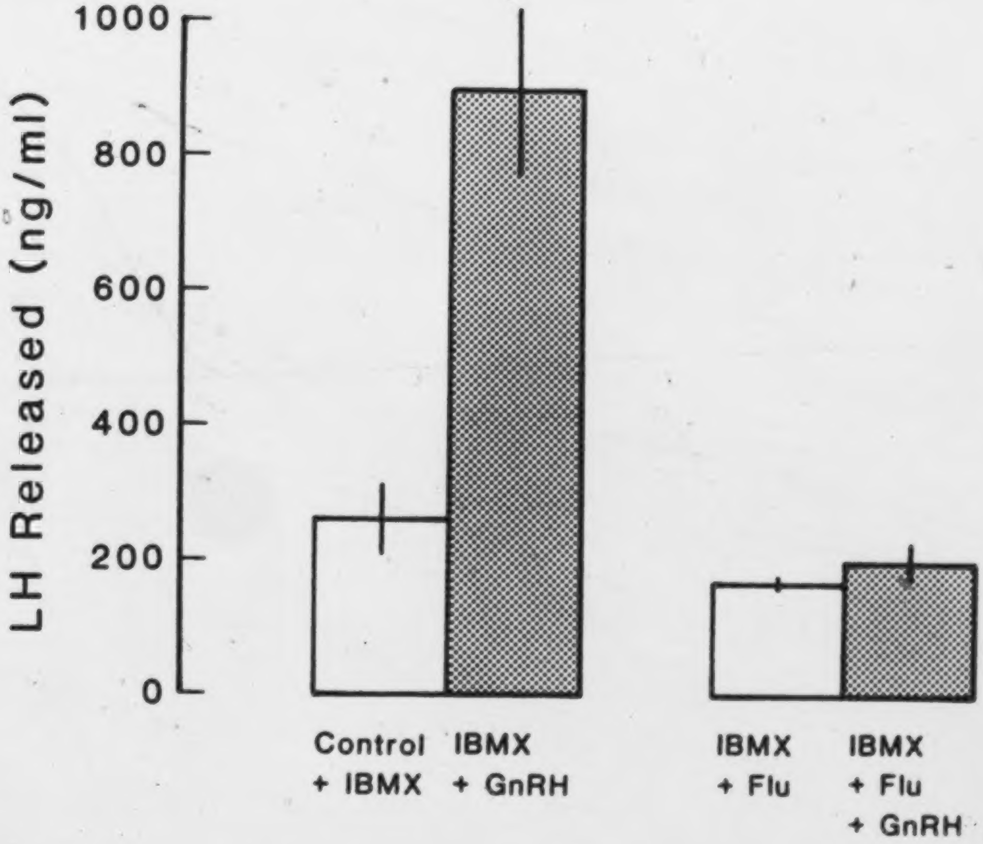
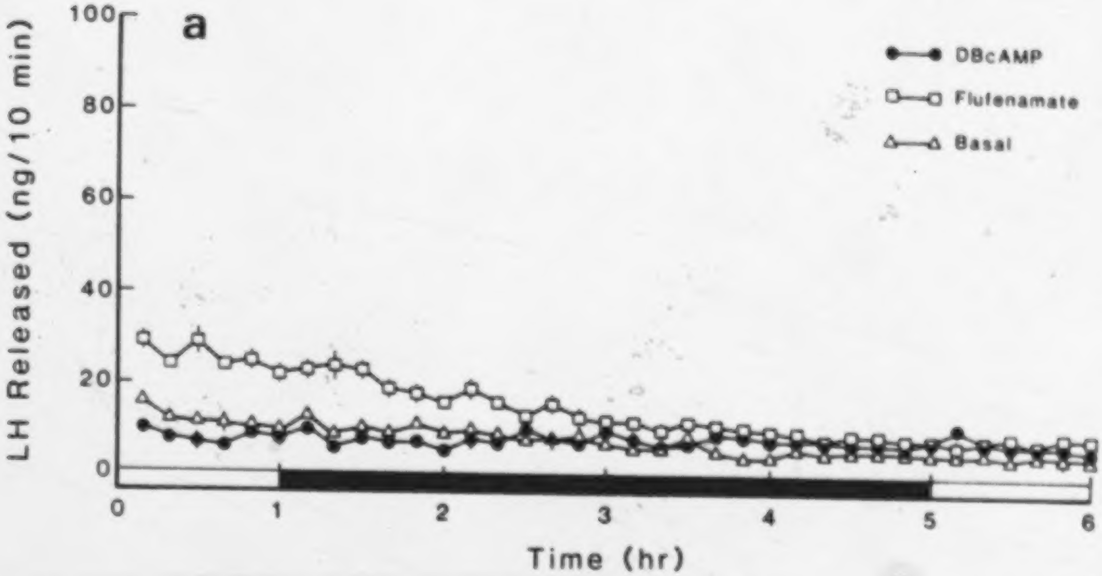

LH Released (ng/ml)
1000
800
600
400
200
0
Control + IBMX
IBMX + GnRH
IBMX + Flu
IBMX + Flu + GnRH

FIG. 22. Basal release and the secretory profiles of LH and FSH obtained from pituitaries of OVX rats in the presence of flufenamate (0.1 mM) or in response to continuous infusions (4 h) of DBcAMP (5 mM). Flufenamate was present in the medium from the beginning of the experiment, while infusions of DBcAMP are indicated by the black bar. Results are means ± SEM (n=3). For further details, see legend for Fig. 4.


a
DBcAMP
Flufenamate
Basal
LH Released (ng/10 min)
100
80
60
40
20
0
Time (hr)
0
1
2
3
4
5
6

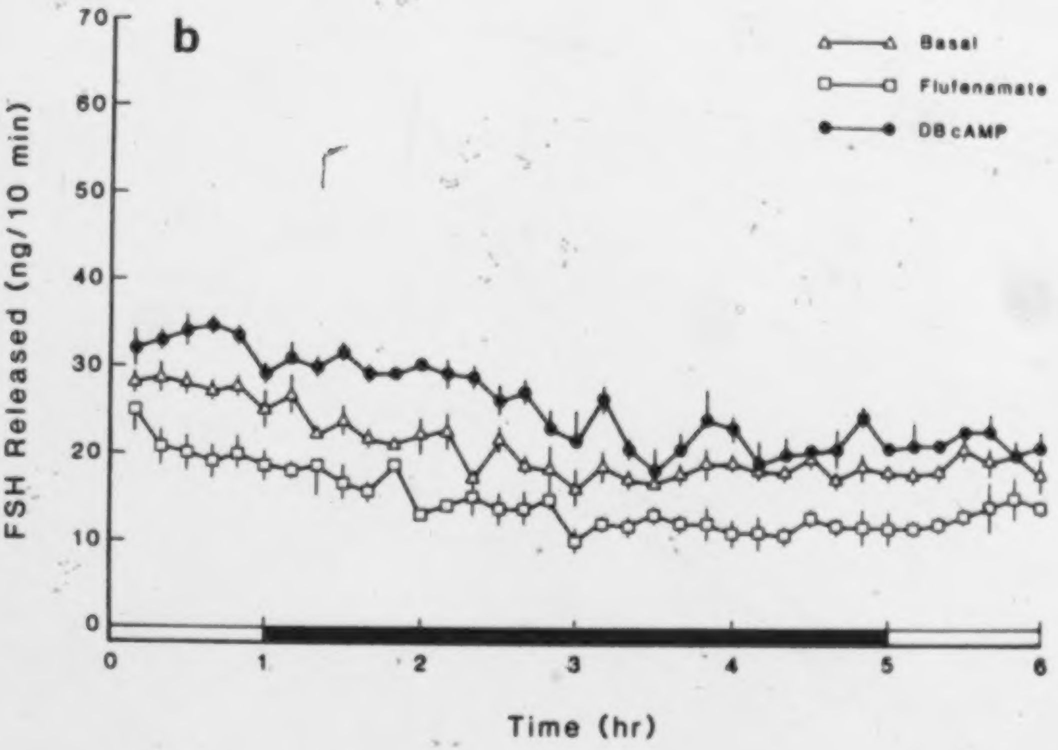

b
Basal
Flufenamate
DBcAMP
FSH Released (ng/10 min)
70
60
50
40
30
20
10
0
Time (hr)
0
1
2
3
4
5
6

FIG. 23. Responses from pituitaries of OVX rats to continuous infusions (4 h) of 1 nM of GnRH and 0.1 mM of flufenamate in the presence or absence of DBcAMP (5 mM) ± cycloheximide (5 μM). Flufenamate and cycloheximide were present in the medium from the beginning of the experiment. Infusions of GnRH and DBcAMP are indicated by the black bar (n=3). For further details, see legend for Fig. 4.

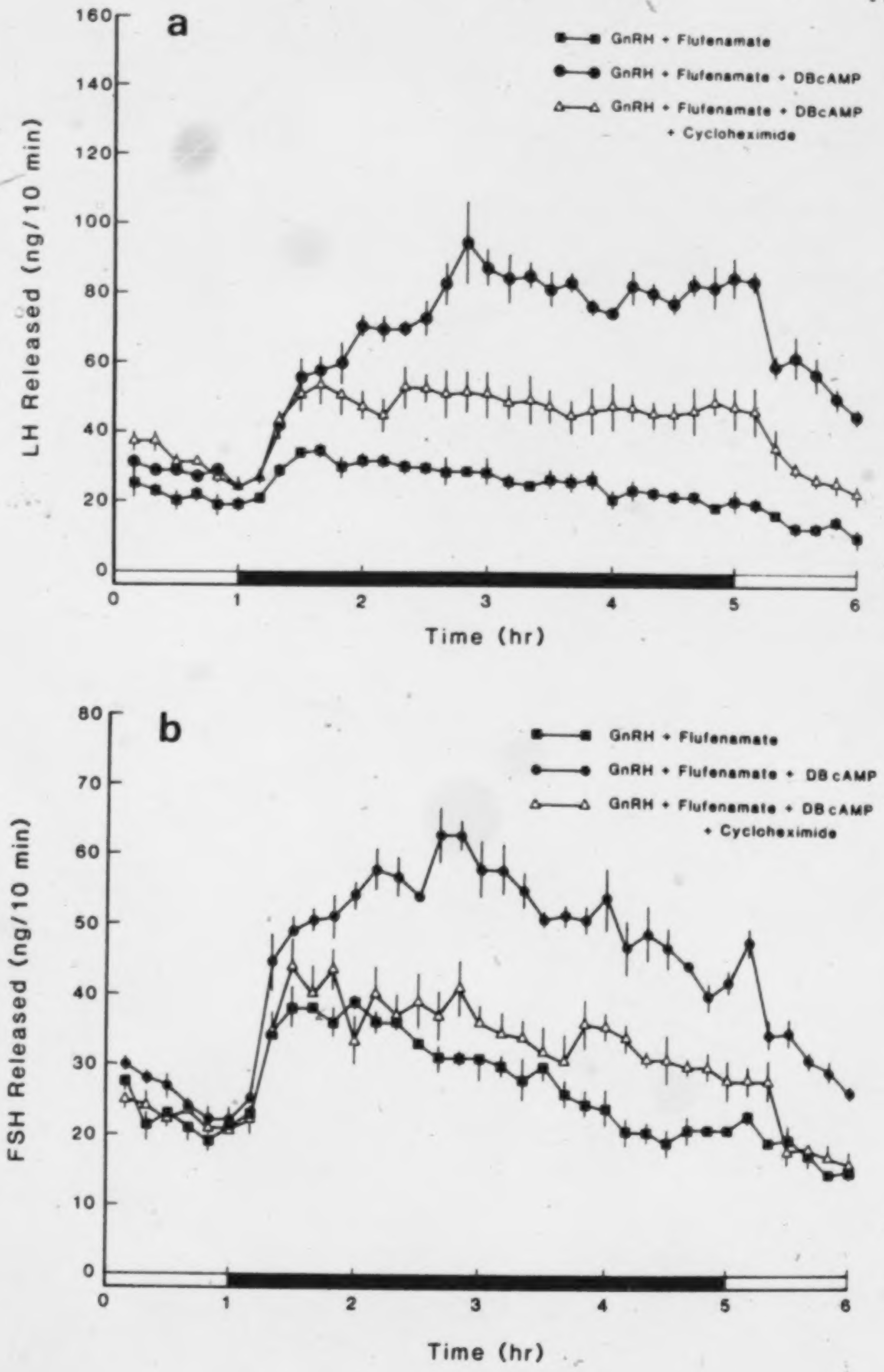

a
GnRH + Flufenamate
GnRH + Flufenamate + DBcAMP
GnRH + Flufenamate + DBcAMP + Cycloheximide
LH Released (ng/10 min)
0
20
40
60
80
100
120
140
160
Time (hr)
0
1
2
3
4
5
6
b
GnRH + Flufenamate
GnRH + Flufenamate + DB cAMP
GnRH + Flufenamate + DB cAMP + Cycloheximide
FSH Released (ng/10 min)
0
10
20
30
40
50
60
70
80
Time (hr)
0
1
2
3
4
5
6

TABLE 1

Total amounts* of LH and FSH released during the first hour of secretagogue infusion in the presence of the indicated pharmacological inhibitors.

Experimental groups	LH (ng)	FSH (ng)
GnRH + Flu	53 ± 8.58	76.67 ± 5.22
GnRH + Flu + DBcAMP	143.21 ± 20.59	122.34 ± 6.94
GnRH + Flu + DBcAMP + Cycloheximide	115.48 ± 7.88	80.73 ± 12.96
GnRH + Flu + Butyrate	63.64 ± 12.28	127.19 ± 8.80
GnRH + Flu + 5'-AMP	90.50 ± 9.80	101.23 ± 18.09

* Values represent the means ± SEM.

All values were corrected for basal secretion.

Abbreviations and statistical data are explained in the text.

The successful stimulation of gonadotropin secretion by DBcAMP resulted in the following experiments being conducted to determine whether the stimulatory effect was specific to cAMP, and/or was due to effects of butyrate. Either butyrate or 5'-AMP were infused together with GnRH and Flu. Neither butyrate nor 5'-AMP was effective in reversing the Flu-inhibited, GnRH-stimulated LH and FSH secretion (Fig. 24[a,b]). The secretory profiles obtained in the presence of these agents were similar to those obtained with GnRH in the presence of Flu (cf. Fig. 23[a,b]).

The total amounts of LH and FSH released in response to the various secretagogues are shown in Fig. 25[a,b]. About 23% of GnRH-stimulated LH and FSH secretion was independent of protein synthesis. Interestingly, total amounts of LH and FSH released in response to GnRH and Flu were not significantly different from those obtained with GnRH and cycloheximide.

The responses obtained in the presence of DBcAMP, GnRH + Flu were quantitatively greater than the responses obtained from GnRH alone (Fig. 25[a,b]). Although cycloheximide inhibited a major portion of the DBcAMP-response, the compound did not reduce the DBcAMP-LH response back to the level obtained in response to GnRH and Flu (Fig. 25[a,b]) i.e. the potentiated effects of DBcAMP on the initial LH response were not affected by 5 µM of cycloheximide (Table 1).

Butyrate administration (in the presence of GnRH and Flu), resulted in LH and FSH responses which were not significantly different from those obtained in response to GnRH and Flu.

FIG. 24. Responses from pituitaries of OVX rats to continuous infusions (4 h) of GnRH (1 nM) and flufenamate (0.1 mM) in the presence of 5'-AMP (5 mM) or butyrate (5 mM). Flufenamate was present in the medium from the beginning of the experiment. Infusions of GnRH, butyrate and 5'-AMP are indicated by the black bar (n=3). For further details, see legend for Fig. 4.

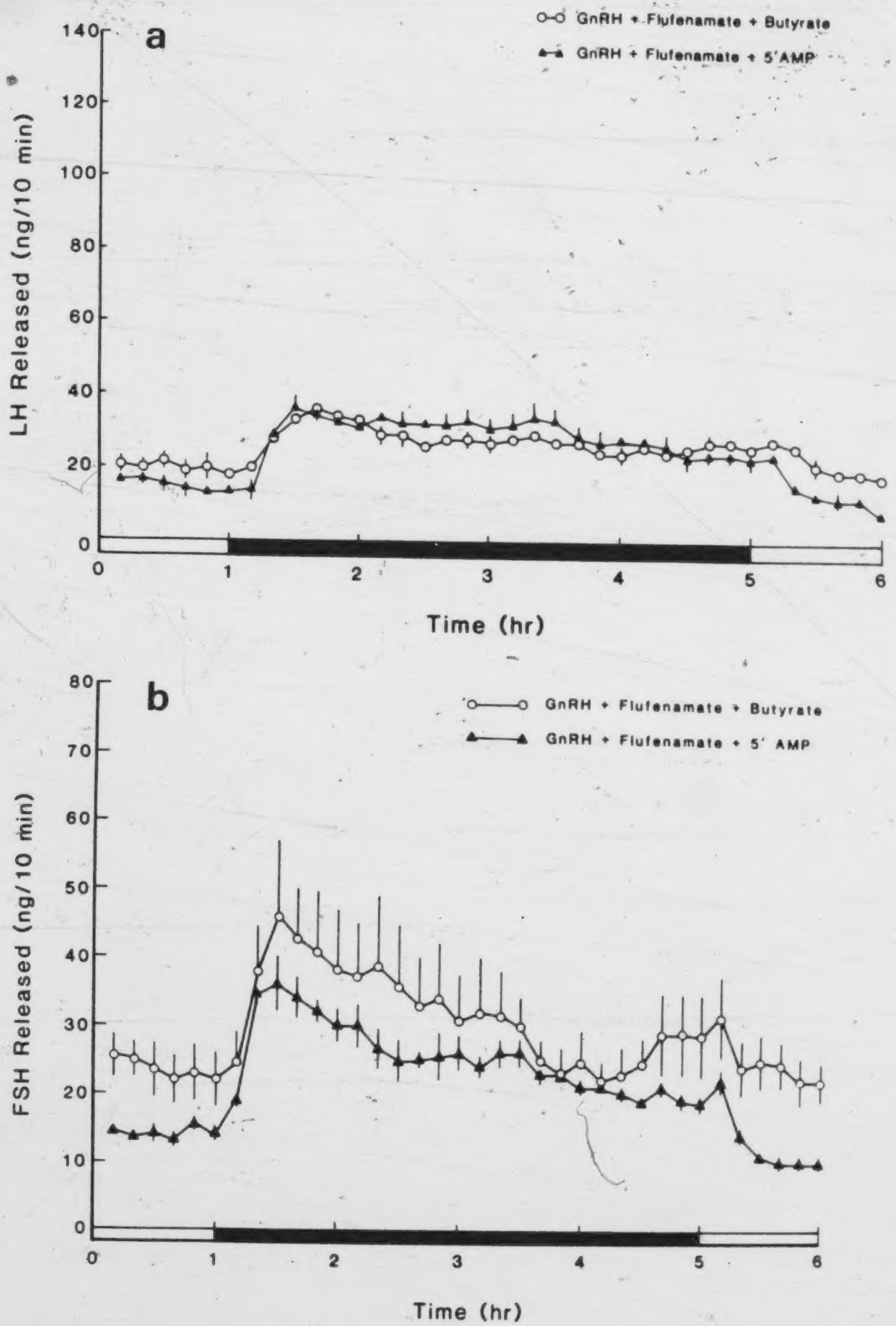

a
GnRH + Flufenamate + Butyrate
GnRH + Flufenamate + 5'AMP
LH Released (ng/10 min)
0
20
40
60
80
100
120
140
Time (hr)
0
1
2
3
4
5
6
b
GnRH + Flufenamate + Butyrate
GnRH + Flufenamate + 5' AMP
FSH Released (ng/10 min)
0
10
20
30
40
50
60
70
80
Time (hr)
0
1
2
3
4
5
6

FIG. 25a. Total amounts of LH released (above basal levels) from pituitaries of OVX rats in response to GnRH (1 nM) in the absence or presence of cycloheximide (C, 5 µM), GnRH + Flu (0.1 mM), GnRH + Flu in presence of DBcAMP (5 mM) ± cycloheximide and GnRH + Flu in the presence of butyrate (5 mM) or 5'-AMP (5 mM). The results are means ± SEM (n=3). Statistical data are included in the text. For further details, see legend for Fig. 11a.

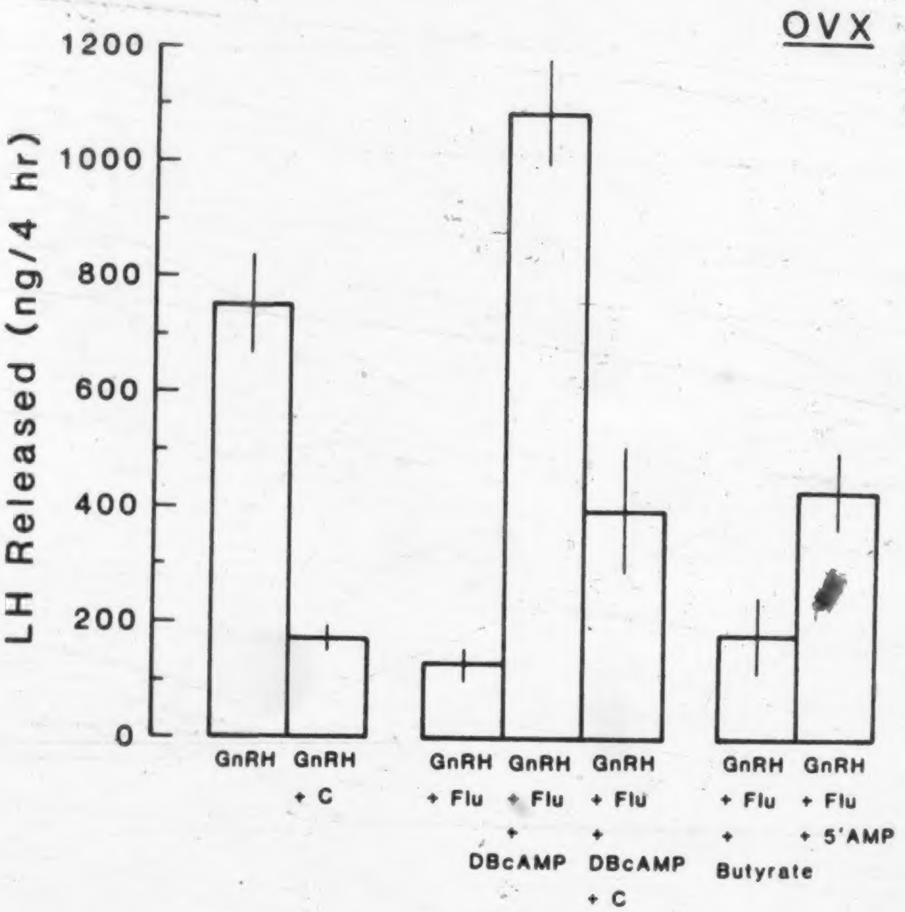
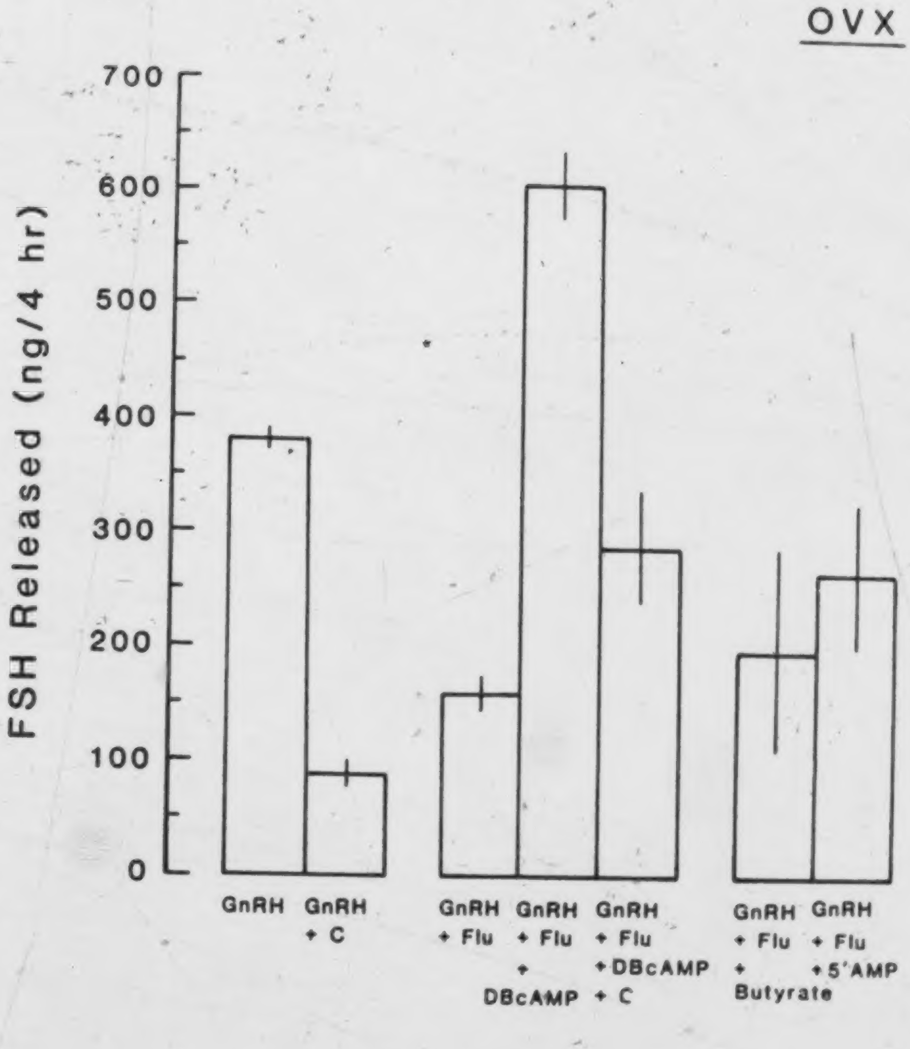

OVX
LH Released (ng/4 hr)
1200
1000
800
600
400
200
0
GnRH
GnRH + C
GnRH + Flu
GnRH + Flu + DBcAMP
GnRH + Flu + DBcAMP + C
GnRH + Flu + Butyrate
GnRH + Flu + 5'AMP

FIG. 25b. Total amounts of FSH released (above basal levels from pituitaries of OVX rats in response to GnRH (1 nM) in the absence or presence of cycloheximide (C, 5 µM), GnRH + Flu (0.1 mM), GnRH + Flu in presence of DBcAMP (5 mM) ± cycloheximide and GnRH + Flu in the presence of butyrate (5 mM) or 5'-AMP (5 mM). The results are means ± SEM (n=3). Statistical data appear in the text. For further details, see legend for Fig. 11a.


OVX
FSH Released (ng/4 hr)
700
600
500
400
300
200
100
0
GnRH
GnRH + C
GnRH + Flu
GnRH + Flu + DBcAMP
GnRH + Flu + DBcAMP + C
GnRH + Flu + Butyrate
GnRH + Flu + 5'AMP

Chapter V

DISCUSSION

As mentioned earlier, the present study was undertaken to delineate the roles/interactions of the branches of the Ca^{++} messenger system as mediators of the biphasic secretion of LH and FSH from pituitaries of diestrous II rats, and document any changes in their roles/interactions following ovariectomy. Additional studies were also undertaken to determine the role(s) of cAMP as a mediator of gonadotropin secretion from OVX rat pituitaries.

An anterior pituitary tissue perifusion system, which maintains gonadotropes' responsiveness for at least 7 h (Bourne and Baldwin, 1980) was chosen as the model system for the successful completion of the objectives (see " Rationale for Experimental Models "). Unfortunately, a disadvantage of this perifusion system is an inherent loss of sensitivity i.e. relatively high concentrations of secretagogues are required to stimulate significant amounts of LH and FSH. The requirement for the increased concentration of secretagogues is due to (i) the use of tissue blocks (with variable diffusion of secretagogues to cells within the block), (ii) loss of material by adsorption to inlet tubings and chambers and (iii) the relatively large dead space of the system due to the retention volumes of tubings and chambers. It should be emphasized, however, that while the concentrations of secretagogues in the medium are high, the effective concentrations of secretagogues affecting the cells are expected to be much lower. Evidence for this supposition will be given in the following section using ionomycin as an example.

A. Effects of Ionomycin and PMA on Gonadotropin Secretion from Pituitaries of Diestrous II Rats

The importance of Ca^{++} as a mediator of GnRH-stimulated gonadotropin secretion was demonstrated in numerous studies (see " Literature Review " section). It is now generally accepted that at least two events occur in gonadotropes when the Ca^{++} messenger system is activated. These are a transient rise in cytosolic Ca^{++} concentrations (Ca^{++} mobilization) (Clapper and Conn, 1985, Chang et al., 1986[a] and Limor et al., 1987), and the activation of phospholipase C which hydrolyzes PIP_2 into DAG and IP_3, increasing the intracellular concentrations of these compounds (Andrews and Conn, 1986). Ca^{++} mobilization results in the activation of the Ca^{++}-calmodulin system (Rasmussen and Barrett, 1984 and Conn et al., 1987[a]), as well as the Ca^{++}-dependent, membrane-binding proteins (Pollard et al., 1987 and Turgeon et al., 1991). On the other hand, the increased concentrations of DAG result in the binding of PKC to the inner surface of the plasma membrane (Hirota et al., 1985 and Naor et al., 1985[b]) where, in the proper mixture of membrane phospholipids, PKC's sensitivity to Ca^{++} is increased, facilitating the formation of a Ca^{++}-PKC complex and subsequent activation of the enzyme (Yamanishi et al., 1983).

Therefore, there are at least 3 branches of the Ca^{++} messenger system: the Ca^{++}-calmodulin branch and the Ca^{++}-dependent, membrane-binding proteins are activated by Ca^{++} mobilization, while the PKC branch is activated by changes in the cellular concentrations of DAG. It is noteworthy that the phorbol esters can substitute for DAG in sensitizing PKC to Ca^{++} such that the enzyme is activated without a rise in cytosolic Ca^{++} (Yamanishi et al., 1983, Nishizuka, 1984[a] and Rasmussen and Barrett, 1984). Equally interesting is the fact that the divalent cation ionophores, A23187 and ionomycin can achieve Ca^{++} mobilization without activating PKC (cf. Rasmussen and Barrett, 1984). Consequently, ionomycin was used in the present study to increase cytosolic Ca^{++}, while the phorbol ester PMA was used for the activation of PKC.

Ionomycin was chosen for these studies because it exhibits a greater selectivity for Ca^{++} than other ionophores (Liu and Hermann, 1978), making it the most effective in stimulating LH secretion (Chang et al., 1986[a]). Although DAG is the endogenous activator of PKC, PMA was used in the present study to activate PKC for the following reasons. DAG was recently shown to stimulate the production of inositol phosphates (IP) in the anterior pituitary (Huckle and Conn, 1987), raising the possibility that exogenously administered DAG might result in IP_3 formation. Since IP_3 was shown to mobilize Ca^{++} from intracellular stores (Naor et al., 1988[a]), its production by DAG could conceivably increase cytosolic Ca^{++} and activate the Ca^{++} mobilization-dependent processes. This could pose significant problems with data interpretation if one makes the assumption that DAG does not affect Ca^{++} mobilization but activates PKC alone. Interestingly, PMA does not suffer from this potential disadvantage, since it does not directly increase IP production (Huckle and Conn, 1987), or affect phospholipid turnover in enriched gonadotropes (Andrews and Conn, 1986).

The ionomycin-induced secretion in the present study was shown to be partially independent of protein synthesis. Additionally, it exhibited similar temporal characteristics to the GnRH-stimulated phase I secretion, which was previously shown to be extracellular Ca^{++}-dependent and protein synthesis-independent (Bourne and Baldwin, 1980). Taken together, these observations suggest that the activation of mechanisms by Ca^{++} mobilization mediates the GnRH-stimulated, phase I secretion of LH and FSH, i.e. Ca^{++} mobilization is intimately involved in the initial responses. As discussed earlier, Ca^{++} mobilization is expected to result in the activation of at least two systems. These are the Ca^{++}-calmodulin system and the Ca^{++}-dependent, membrane-binding proteins. As a result, both calmodulin and synexin and/or other annexins are expected to play a role in mediating the phase I secretion. Direct evidence for the involvement of calmodulin or the synexins/annexins will be dependent on the discovery of potent and highly specific antagonists to these proteins. In this regard, it is interesting to note that a number of the so-called calmodulin antagonists also inhibit synexin's interactions with plasma membranes (Pollard et al., 1987).

The demonstration that Ca^{++} mobilization mediates phase I secretion in gonadotropes signifies that gonadotropes are somewhat analogous to other endocrine cells in which the initial responses were shown to be stimulated by ionophores (Kojima et al., 1983 and Zawalich et al., 1983). However, one difference between the gonadotropes and the other endocrine cells is the fact that the initial responses in the other studies were transient (Kojima et al., 1983 and Zawalich et al., 1983), whereas in the present study the ionomycin-induced initial response was sustained.

The fact that phospholipase C activation appears to be independent of Ca^{++}, and ionophores do not affect phospholipid turnover in the anterior pituitary (Naor et al., 1985[a], Andrews and Conn, 1986 and Huckle and Conn, 1987), suggests that the phase I secretion occurs independently of PKC. As a result, it appears that the GnRH-stimulated phase I secretion of gonadotropins is mediated by mechanisms activated by Ca^{++} mobilization, namely, Ca^{++}-calmodulin and Ca^{++}-dependent, membrane-binding proteins. Additionally, this component was previously shown to be dependent on extracellular Ca^{++} and independent of protein synthesis and cAMP (Bourne and Baldwin, 1980, 1987[a]).

The inability of ionomycin to completely mimic GnRH actions (with respect to the kinetics and the amount of LH and FSH secreted) in the present study is at variance with other reports (Conn et al., 1979[b], 1987[c]), but agrees with the study by Naor et al., (1988[a]). In the study by Naor et al. (1988[a]), ionomycin accounted for only 45% of the GnRH-stimulated LH response even though the ionophore elevated gonadotrope intracellular Ca^{++} levels to the same concentrations induced by GnRH. As a result, it is plausible that the discrepancies between these studies (my study and Naor's study vs Conn's study) could be due to Conn's use of high concentrations of ionophores (0.1 mM) (Conn et al., 1979[b]), which increased intracellular Ca^{++} to levels which were much greater than those achieved by GnRH.

Although relatively high concentrations of ionomycin (10 μM) were used in the present study (reasons for this were discussed previously), it is believed that cytosolic Ca^{++} concentration induced in gonadotropes by 10 μM ionomycin is less than that achieved by 1 nM GnRH. This conclusion is based on the fact that when ionomycin increased Ca^{++}

mobilization in gonadotropes to the same extent as GnRH, only 45 % of the GnRH-stimulated LH response was evident (Naor et al., 1988[a]). In the present study, about 21.5 % of the 1 nM GnRH-stimulated LH response was obtained by 10 μM ionomycin, suggesting that cytosolic Ca^{++} concentrations due to ionomycin were lower than the concentrations achieved by 1 nM GnRH. As a result, potentially adverse pharmacological effects due to high concentrations of mobilized cytosolic Ca^{++} by ionomycin are not expected to pose any significant problems in the present study. One potentially adverse effect which is of paramount importance to the present study is the possibility that high concentrations of cytosolic Ca^{++} could activate PKC (in the absence of DAG / PMA) (Nishizuka, 1984[b]). However, the observations discussed above, coupled with the fact that the characteristics of the secretory profiles due to ionomycin are distinctly different from those obtained in response to PMA (i.e. ionomycin does not activate PMA-stimulated mechanisms), provides further evidence that the concentration of ionomycin used in this perifusion system does not produce this unwanted effect.

The secretory profiles of LH and FSH obtained in response to PMA in the present study suggest that PMA-activated mechanisms might mediate part of the second phase release of the gonadotropins, as evident by the dependency on protein synthesis and the delayed onset of secretion. This observation, coupled with the report that PMA stimulates LH secretion from pituitaries of diestrous II rats perifused with Ca^{++}-free KIRB medium (Bourne et al., 1989), suggests that PMA-stimulated mechanisms might mediate the GnRH-stimulated phase II extracellular Ca^{++}-independent component of LH and FSH secretion. As reported previously, this component is also indirectly dependent on cAMP (Bourne, 1988).

Although some doubts were expressed concerning the essentiality of PKC as a mediator of GnRH-stimulated LH secretion (McArdle et al., 1987 and Conn, 1989), the results of the present report suggest that PKC may play a role in the GnRH-stimulated gonadotropin secretion. However, it should be emphasized that while PMA does activate

PKC in anterior pituitaries (Hirota et al., 1985), the potential side effects of this non-endogenous, pharmacological agent cannot be excluded, i.e. gonadotropin secretion may be due to other effects of PMA. Obviously, the demonstration of PKC's involvement in GnRH-stimulated gonadotropin secretion will be dependent on the availability of extremely potent and specific inhibitors of PKC. The fact that PMA-induced gonadotropin secretion in the absence of extracellular Ca^{++} mimics all of the characteristics of the GnRH-stimulated response (Bourne et al., 1989) suggests that the mechanisms activated by PMA might also be involved in GnRH-stimulated gonadotropin secretion. In other words, whatever mechanisms (PKC and/or otherwise) are activated by PMA, these same mechanisms are expected to be involved in some of the releasing actions of GnRH.

The results obtained from the administration of PMA and ionomycin together suggest that the simultaneous activation of the Ca^{++} mobilization- and PMA-activated mechanisms stimulate a biphasic secretion of LH and FSH. Additionally, the results also indicate that part of the second phase secretion of the gonadotropins is the direct result of synergistic interactions between Ca^{++} mobilization- and PMA-activated mechanisms. While synergistic interactions between Ca^{++}-calmodulin and PKC (activated by ionophores and phorbol esters, respectively), were previously reported for gonadotropes (Harris et al., 1985, Naor and Eli, 1985, Davidson et al., 1987 and Wooge and Conn, 1987), to my knowledge this is the first report which localizes the synergism to a specific component of the phase II secretion of gonadotropins. This component was previously shown to be dependent on protein synthesis and extracellular Ca^{++}, and indirectly dependent on cAMP (Bourne and Baldwin, 1980, 1987[a]). The specific mechanisms which might be involved in the synergistic interactions will be discussed in the following section.

The protein synthesis-dependent synergism which occurs between Ca^{++} mobilization-induced mechanisms and PMA-activated systems may offer a possible explanation for the fact that part of the ionomycin-induced LH and FSH secretion is dependent on protein synthesis. It is conceivable that the protein synthesis-dependent secretion of gonadotropins may represent some synergistic interactions between the *activated* Ca^{++} mobilization-induced mechanisms and the *unstimulated*, low basal activity of PKC and/or other PMA-induced mechanisms.

The fact that the simultaneous administration of Ca^{++}-ionophores and phorbol esters stimulates a biphasic gonadotropin response raises some intriguing inferences. Since the phase II secretion was previously shown to be indirectly dependent on cAMP (Bourne and Baldwin, 1987[a]), PMA and/or ionomycin would be expected to increase cAMP production. In fact, preliminary experiments indicate that both ionophores and PMA are capable of increasing cAMP production in anterior pituitaries from males (cAMP levels were 17.3±0.8 pmol [control], 31.2±1.2 pmol for the ionophore, and 30.0±0.4 pmol for PMA [Bourne, unpublished data]). Experiments are underway to determine whether these agents will be equally effective in increasing cAMP production in pituitaries and enriched gonadotrope cell fractions of females.

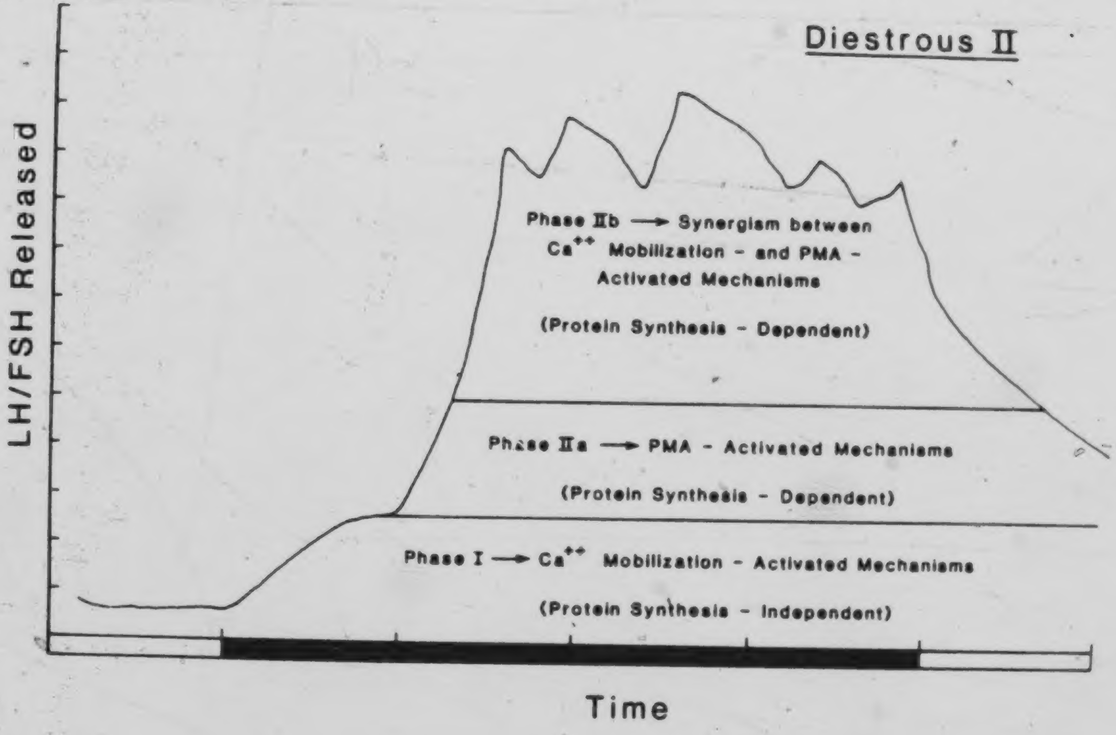
Additionally, a number of reports have implicated the lipoxygenase metabolites of arachidonic acid as mediators of GnRH-stimulated secretion (Chang *et al.*, 1986[b], 1987 and Naor, 1987). If these metabolites do play a role in the biphasic secretion of gonadotropins, and if the simultaneous activation of Ca^{++} mobilization- and PMA-activated mechanisms faithfully reproduces the effects of GnRH, then either or both of these systems would be expected to increase the lipoxygenase metabolites of arachidonic acid. In this regard it is interesting that phospholipase A_2, which releases arachidonic acid from membrane phospholipids, can be activated by Ca^{++}-calmodulin (Rassmussen and Barrett, 1984). These observations, coupled with the demonstration that arachidonic acid and its metabolites which have *cis* unsaturated double bonds (lipoxygenase products) enhance the interactions between Ca^{++}-dependent membrane-binding proteins and cell membranes (Pollard *et al.*, 1987 and Burgoyne and Geisow, 1989), would imply that the lipoxygenase metabolites may be involved in either phase I secretion and/or the Ca^{++}

mobilization-activated mechanisms which participate in the synergistic component of phase II.

Clearly, the biphasic release of gonadotropins is a complex process which appears to be a composite of a number of secretory processes. The realization of the complexity of this secretory process, coupled with the number of implicated intracellular mediators of the actions of GnRH, resulted in the postulate that GnRH-stimulated gonadotropin secretion is the result of a complex interaction of a number of second messengers (Turgeon and Waring, 1986). The results of the present study, coupled with previous reports (Bourne and Baldwin, 1980, 1987[a], Turgeon and Waring, 1986, Chang et al., 1987, Bourne, 1988 and Bourne et al., 1988, 1989) definitely provide evidence which is consistent with this viewpoint.

In conclusion, the present results suggest that the GnRH-stimulated biphasic secretion of gonadotropins can be dissociated into the following components (Fig. 26). The phase I secretion of gonadotropins might be mediated by Ca^{++} mobilization-activated mechanisms (such as Ca^{++}-calmodulin, and Ca^{++}-dependent, membrane-binding proteins). This process(es) appears to be protein synthesis-independent. On the other hand, a component of the protein synthesis-dependent phase II secretion is mediated by PMA-stimulated mechanisms (possibly PKC), while the processes stimulated by Ca^{++} mobilization and PMA synergize to mediate the remaining component of the second phase of gonadotropin secretion.

FIG. 26. Summary of the biphasic secretion of LH/FSH from pituitaries of diestrous II rats, indicating the various components and the branches of the Ca^{++} messenger system which mediates them.


Diestrous II
LH/FSH Released
Phase IIb → Synergism between Ca++ Mobilization - and PMA - Activated Mechanisms
(Protein Synthesis - Dependent)
Phase IIa → PMA - Activated Mechanisms
(Protein Synthesis - Dependent)
Phase I → Ca++ Mobilization - Activated Mechanisms
(Protein Synthesis - Independent)
Time

B. Effects of Ionomycin and PMA on Gonadotropin Secretion from Pituitaries of OVX Rats

The results of the initial experiments undertaken to characterize LH and FSH secretion in response to GnRH indicated a distinct difference in the profiles of LH and FSH obtained from pituitaries of OVX rats when compared to those observed from pituitaries of diestrous II rats. In accordance with previous observations (Baldwin et al., 1983), ovariectomy resulted in an attenuation of the amounts of LH and FSH secreted. Furthermore, the inhibition of a predominant portion of the gonadotropin secretion by cycloheximide suggests that a large component of the GnRH-stimulated secretion is protein synthesis-dependent, while a small but significant portion is protein-synthesis-independent. It appears that the small, protein synthesis-independent component is an expression of the phase I component of the biphasic secretion that is evident from pituitaries of diestrous II rats, while the larger, protein synthesis-dependent component represents part of the phase II secretion. Interestingly, the GnRH-stimulated gonadotropin response observed from pituitaries of OVX rats resembled the GnRH-stimulated responses from pituitaries of male rats (Bourne and Baldwin, 1987[b] and Fahmy, 1990).

The release of LH and FSH obtained in response to ionomycin indicates that Ca^{++} mobilization and subsequent activation of Ca^{++}-calmodulin and/or the Ca^{++}-dependent, membrane-binding proteins probably play a role in stimulating the acute, gonadotropin release from pituitaries of OVX rats. Furthermore, the inability of cycloheximide to inhibit this ionomycin-induced LH response also indicates that this Ca^{++}-mobilized component is protein-synthesis independent. These results are in excellent agreement with those reported for pituitaries of male rats (Fahmy, 1990), but conflict with the ionomycin-induced responses obtained from pituitaries of diestrous II rats. The latter responses were partially dependent on protein synthesis.

It was previously suggested (see Section V.A) that the protein synthesis-dependent component of ionomycin-stimulated LH secretion from pituitaries of diestrous II rats might be due to synergistic interactions that involved *basal* PKC activity. However, PKC activity in anterior pituitaries is reduced following ovariectomy (Drouva *et al*., 1990). Apparently, the decrease in estradiol levels after ovariectomy results in reduced PKC activity. The reduction in basal PKC activity could result in the absence of the protein synthesis-dependent, synergistic interactions during ionomycin infusions to pituitaries from OVX rats, thereby accounting for the absence of an effect of cycloheximide on the ionomycin-induced secretion.

Interestingly, in contrast to the results obtained for LH, the ionomycin-mediated FSH secretion from pituitaries of OVX rats was protein synthesis-dependent, since a major component of the secretion was inhibited by cycloheximide. This is probably a reflection of the fact that for FSH, phase I secretion is partially dependent on protein synthesis (Bourne and Baldwin, 1987[a]).

The inability of the phorbol ester, PMA, to stimulate gonadotropin secretion from pituitaries of OVX rats was similar to that observed for male pituitaries (Fahmy *et al*., 1989), but different from the responses obtained from pituitaries of diestrous II rats (*cf*. Section V. A.). Studies by Fahmy *et al*., (1989) indicated that the ability of the phorbol ester to directly stimulate LH secretion was dependent on estradiol. Other investigators have also shown that ovariectomy results in a reduction of PKC activity, while the *in vivo* administration of estradiol (over a period of 24 h to 5 days) increased PKC activity in both soluble and particulate fractions prepared from pituitaries of OVX rats (Drouva *et al*., 1990). Additionally, estradiol administration to pituitary cell cultures dramatically increased phorbol ester-stimulated LH release (Liu and Jackson, 1987 and Drouva *et al*., 1990). Taken together, these observations suggest that the decrease in estradiol levels after ovariectomy probably causes a decrease in PKC activity in pituitaries of OVX rats, resulting in an inability of gonadotropes to respond to PMA (if the PMA-stimulated gonadotropin secretion is in fact due to PKC). It should be emphasized that ovariectomy resulted in a decrease of PKC activity, but did not cause a total inhibition of the activity of the enzyme (Drouva et al., 1990).

A possible explanation for the inability of pituitaries obtained from OVX rats to respond to PMA could be the reduced PKC activity resulting from the decreased concentration of circulating estradiol (Drouva et al., 1990). The estradiol-dependent changes in PKC activity could be achieved through two mechanisms. In the first instance, estradiol could stimulate PKC biosynthesis, thereby altering the concentration of PKC, and hence, its activity. Indeed, this possibility was postulated by Drouva et al., (1990). Obviously, the decreased estradiol levels evident after ovariectomy would result in decreased PKC concentrations and ultimately decreased PKC activity.

The second possiblity is based on the demonstration of endogenous PKC inhibitors (Kraft and Anderson, 1983, Turgeon et al., 1984, Naor et al., 1985[b] and Eyster, 1990[a,b]). Conceivably, estradiol could override the inhibitory effects of the endogenous inhibitors by i) stimulating the synthesis of proteins which neutralize the effects of the inhibitors and/or ii) stimulating the biosynthesis of proteins which regulate (decrease) the concentrations/activity of the inhibitors. In either case, decreases in estradiol concentrations would result in an increased activity of the inhibitors and a subsequent decrease in PKC activity. It should be noted that the possiblities described above are not mutually exclusive, and the decrease in PKC activity associated with the reduction in estradiol concentrations could be the result of decreased PKC concentrations and increased activity of the endogenous inhibitors of PKC.

The involvement of PKC as a mediator of GnRH-stimulated gonadotropin secretion was questioned by a number of investigators (McArdle et al., 1987, Beggs and Miller, 1989 and Conn, 1989). These conclusions were based on experiments in which cultured pituitary cells were depleted of PKC activity following pretreatment with PMA. Unfortunately, the PKC depletion was not complete and the cells did secrete small but significant amounts of LH in response to PMA (McArdle et al., 1987). When the PKC-depleted and non-depleted cells were exposed to GnRH, both groups secreted similar amounts of LH (McArdle et al., 1987) suggesting that PKC was probably not involved in GnRH-stimulated LH secretion. However, Stojilkovic et al. (1988) reported that while PKC-depleted cells did respond to GnRH, the magnitude of the LH response was less than the response from non-depleted cells. This observation suggested an involvement of PKC in GnRH-stimulated LH secretion. The difference in results from the two studies appears to be due to the methods of calculating LH secretory responses (Stojilkovic et al., 1988). McArdle et al. (1987) reported LH responses as a percentage of cellular LH present at the beginning of the experimental incubation (3 h) with GnRH. This method of calculation did not take into account the fact that PMA and GnRH stimulated LH biosynthesis, and this significantly contributed to the LH release response (Stojilkovic et al., 1988). As a result, the expression of the results in this fashion would not provide an accurate assessment of the response. In contrast, Stojilkovic et al., (1988) expressed the LH response as a percentage of total LH available (amount secreted and amount remaining in the cell) and were able to demonstrate a PKC-dependent component of LH secretion.

The results of my experiments also provide some insight into the way that cells with low PKC activity could still respond to GnRH with a relatively large gonadotropin response. The small LH secretion obtained from PKC-depleted cells in response to PMA (McArdle et al., 1987) can be compared to the absence of gonadotropin secretion in response to PMA in my study of OVX rats. However, my studies clearly demonstrate that the low PKC activity (demonstrated by the absence of a PMA-activated response) could still synergise with mechanisms activated by Ca^{++} mobilization to give an augmented response (cf. Fig.16[a,b] and Fig.18[a,b]). Indeed, Conn (1989) later speculated that the presence of approximately 10% of PKC activity, found in the particulate fraction of PKC-depleted cells, may be sufficient to evoke a full or near-full response to GnRH. The results of my study clearly demonstrate that this can in fact occur as a result of synergistic interactions with the mechanisms activated by Ca^{++} mobilization.

As discussed above, ionomycin and PMA interacted synergistically to stimulate gonadotropin secretion. The PMA- and ionomycin-induced synergistic component of LH and FSH secretion was dependent on de novo protein synthesis. This fact, coupled with the temporal characteristics of the responses, suggests that a similar synergistic component might be involved in the protein synthesis-dependent gonadotropin secretion stimulated by GnRH. Similar observations and conclusions were reported for pituitaries obtained from males, where PMA, in conjunction with ionomycin, stimulated gonadotropin release in a synergistic manner, in spite of the fact that the phorbol ester by itself was an ineffective secretagogue (Fahmy, 1990). Presumably, the combined effects of Ca^{++} mobilization and the nominal PMA-stimulated PKC activity were sufficient for the manifestation of the synergistic response.

The exact cellular mechanisms underlying the synergism are presently unknown but are clearly dependent on de novo protein synthesis. The mechanisms involved may include the following: (1) ionomycin and/or PMA may recruit other intracellular mediators, e.g. cAMP and/or the AA metabolites of the lipoxygenase pathway. Ionomycin-induced Ca^{++} mobilization results in the activation of a number of systems, one of them being calmodulin. Since Ca^{++}-calmodulin activates phospholipase A_2 in a number of systems (Rasmussen and Barrett, 1984), it is conceivable that similar responses could occur in gonadotropes. This would result in AA production from membrane phospholipids, and subsequent generation of the lipoxygenase metabolites. These compounds could then enhance exocytosis through their effects on the functions of the Ca^{++}-dependent, membrane-binding proteins (see " Literature Review "). Moreover, as discussed in the last section, both ionomycin and PMA might stimulate cAMP production, and recruit the cyclic nucleotide and its potentiating effects which are associated with protein synthesis. The recruitment of these and/or other intracellular mediators could result in augmented responses forming the basis for the synergistic interactions. (2) Ionomycin may cause the activation of certain effector proteins associated with gonadotropin secretion, while PMA may activate others. As a result, the combination of these secretagogoues may result in the simultaneous activation of all of the proteins, generating a synergistic response. An analogy of this situation can be drawn from the release reaction of platelets. Thrombin stimulates a release reaction which is associated with the phosphorylation of two proteins with approximate masses of 20 kDa and 40 kDa (Nishizuka, 1984[b]). The 20 kDa protein serves as a substrate for a calmodulin-dependent protein kinase and absolutely requires Ca^{++} mobilization for its activation, while the 40 kDa protein is phosphorylated by PKC (Nishizuka, 1984[b]). When secretagogues are administered to activate both calmodulin (ionomycin) and PKC (PMA), both proteins are phosphorylated resulting in a synergistic response (Kaibuchi et al., 1982, 1983 and Nishizuka, 1984[b]). (3) Another possibility involves the endogenous inhibitors of PKC. It is conceivable that activation of the Ca^{++}-calmodulin system might result in the neutralization of the effects of the endogenous inhibitors of PKC. Consequently, Ca^{++} mobilization would result in the full expression of the PMA-stimulated mechanisms, with augmented synergistic responses being evident. (4) Finally, the synergistic interactions could be due to the ability of PMA to sensitize the effector mechanisms to the actions of calcium. The annexins, which presumably are involved in exocytosis, are activated by Ca^{++} (Burgoyne and Geisow, 1989 and Turgeon et al., 1991). Moreover, the annexins in the anterior pituitary contain sites for PKC-mediated phosphorylation (Turgeon et al., 1991). It is therefore conceivable that phosphorylation of the annexins after activation by Ca^{++} could modify their activity, resulting in augmented responses. Indeed, PMA-stimulated PKC was shown to increase the responsivness of the exocytosis process to calcium in adrenal medullary cells (Knight and Baker, 1983). The increased sensitivity resulted in an increased secretory response. Since all of the mechanisms described above are not mutually exclusive, it is possible that some or all of these mechanisms may play a role in mediating the synergistic interactions of ionomycin and PMA.

In contrast to the biphasic LH secretory rates obtained (in response to GnRH or ionomycin plus PMA) from pituitaries of OVX rats, FSH exhibited an apparent uniphasic response suggesting the presence of a single "mean" secretory rate following ovariectomy. However, cycloheximide in the presence of GnRH or ionomycin plus PMA, dissociated the FSH response into protein synthesis-dependent and -independent components. These results suggest that the protein synthesis-dependent component is indicative of phase II secretory mechanisms, while the protein systhesis-independent component represents phase I mechanisms i.e. biphasic mechanisms are still operative. It may thus be speculated that following ovariectomy, the "mean" rate of FSH secretion in the attenuated, protein synthesis-dependent second phase is reduced to a level equal to or less than the "mean" rate of secretion of the phase I responses, generating an apparent uniphasic rate of secretion. If the suggestion is true, then obviously there is a differential effect on the degree of attenuation of FSH phase II responses when compared to LH responses. This differential effect on FSH secretion in comparison to LH secretion could be due to paracrine inhibitory effects of 'follistatin' which is secreted by the folliculo-stellate cells of the pituitary (see " Literature Review ", Gospodarowicz and Lau, 1989).

The overall conclusion emerging from the above studies is that the attenuated GnRH-stimulated gonadotropin secretion from pituitaries of OVX rats is a composite of two major secretory components. It consists of an initial, protein synthesis-independent component activated by Ca^{++}-mobilization, and a second, protein synthesis-dependent component that is due to synergistic interactions of Ca^{++}-mobilization- and PMA- activated mechanisms (Fig. 27). It is tempting to speculate that the protein synthesis-independent component might be equivalent to the phase I component obtained from pituitaries of diestrous II rats, while the protein synthesis-dependent component might be a remnant of the phase II secretion observed from diestrous II animals. Finally, the attenuation of the second phase of the biphasic gonadotropin response following ovariectomy is due (at least in part) to the absence of the PMA-stimulated (possibly PKC-mediated) component of the second phase.

C. Role of Cyclic AMP in GnRH-Stimulated Gonadotropin Secretion from pituitaries of OVX Rats

Previous reports from this laboratory have indicated a stimulatory effect of GnRH on cAMP production in pituitaries obtained from both male and female rats (Bourne and Baldwin, 1987[a,b]). The current study extends these observations on GnRH-stimulated cAMP production and demonstrates that GnRH does increase intracellular cAMP concentrations in pituitaries from OVX rats, albeit only in the presence of IBMX. The requirement for a phosphodiesterase inhibitor to demonstrate this effect in pituitaries of OVX rats is surprising in view of the fact that IBMX was not required to observe the effect in pituitaries obtained from intact female rats (Bourne and Baldwin, 1987[a]). Obviously, it appears that ovarian factor(s) affect the adenylyl cyclase-cAMP system. The absence of the ovarian factor(s) following ovariectomy could result in an increased PDE activity and/or decreased adenylyl cyclase activity such that PDE inhibition is required for the observation of the GnRH effect on cAMP production. In this regard, it is interesting to note that estradiol stimulated cAMP production in monolayer cultures of cells dispersed from pituitaries of females (Tang et al., 1982) suggesting that decreases in estradiol could result in decreased cAMP production, thereby giving some credence to these possibilities.

The concentration of GnRH (75 nM) used in the static incubation system is greater than that (1 nM) used in the perifusion system. The use of the higher concentration of GnRH in static incubations is based on the fact that the decapeptide is easily degraded by enzymes in pituitary cell membranes (Clayton et al., 1979[b]). This results in the progressive reduction of GnRH concentrations throughout the experimental incubation. The relatively high initial concentrations ensure that an effective concentration of GnRH is still present at the end of the experimental incubation. In contrast, the perifusion system obviously does not suffer from this problem since as a dynamic system there is a continuous replenishment of GnRH.

The use of Flu in determining the role of cAMP as a mediator of GnRH action was based on the fact that Flu inhibits GnRH-stimulated increases in cAMP production (Naor *et al.*, 1975[a,b] and Bourne and Baldwin, 1987[a,b]). Unfortunately, Flu was not expected to affect gonadotropes only. However, gonadotropes apparently are the only cell types in the anterior pituitary which have GnRH receptors (Clayton and Catt, 1981). This in turn suggests that the effects of Flu on GnRH-stimulated cAMP production probably occur in gonadotropes. However, it should be emphasized that other cell types might still be involved, *e.g.*, mammotropes through their close association with gonadotropes (see "Literature Review") and/or paracrine effects of other cells, such as, folliculo-stellate cells.

The initial results indicate that Flu inhibited the GnRH-stimulated increases in cAMP production in pituitaries from OVX rats. The exact mechanism(s) by which Flu inhibits GnRH-stimulated increases in cAMP production remain unknown. While the drug is a known cyclooxygenase inhibitor (Flowers *et al.*, 1980), the inhibitory effects on prostaglandin synthesis are not related to its inhibitory effects on cAMP production, since other potent prostaglandin synthesis inhibitors did not affect GnRH-stimulated cAMP production in anterior pituitaries (Naor *et al.*, 1975[b]). Recent experiments from this laboratory, however, have begun to shed some light on this problem. Flu does not affect GnRH-receptor interaction as evidenced by its inability to significantly affect either the affinity or binding capacity of GnRH (Bourne, unpublished data). Moreover, Flu inhibited cholera toxin-stimulated cAMP production, but was ineffective against forskolin-induced activation of adenylyl cyclase (Bourne, unpublished data). Since GnRH and cholera toxin activate adenylyl cyclase via the GnRH receptor and the G_s protein, respectively, and forskolin exerts its stimulatory effects via the catalytic component (adenylyl cyclase) directly (Berridge, 1985), the data suggest that the inhibitory effects of Flu on adenylyl cyclase activation occur at the level of the G_s protein, prior to its involvement in the activation of the catalytic component.

As mentioned above, the actions of Flu are not specific. In addition to inhibiting cAMP production, it is also a known cyclooxygenase inhibitor (Flowers et al., 1980). Some compounds, which inhibit the cyclooxygenase enzyme, also inhibit the lipoxygenase enzyme when administered in high dosages. This raises the possibility that the inhibitory effects of Flu on LH and FSH secretion may be due to the absence of the lipoxygenase metabolites and not due to cAMP. However, this possibility is greatly minimized by the fact that DBcAMP which does not stimulate gonadotropin secretion by itself, does overcome the inhibitory effects of Flu, suggesting that the effects of Flu are associated with its effects on the adenylyl cyclase-cAMP system.

In addition to inhibiting GnRH-stimulated cAMP production, Flu also completely inhibited GnRH-stimulated LH secretion in the static incubation experiments. While Flu inhibited a major component of gonadotropin secretion in the perifusion experiments above, a small but significant component of LH and FSH secretion was still apparent. Unfortunately, the discrepancy in the results obtained from the two systems cannot be easily explained at present, but it may reflect paracrine and/or negative feedback effects of accumulated hormones and/or metabolic by-products in the static system.

The small amounts of LH and FSH released in response to GnRH in the presence of Flu are not significantly different from the responses obtained with GnRH in the presence of cycloheximide (Fig.25[a,b]). Presumably the initial, small, protein synthesis-independent component of gonadotropin secretion is not mediated by cAMP. An extrapolation of results from the previous section also indicates that this component is mediated by the mechanisms activated by Ca^{++} mobilization. Thus, the initial release of gonadotropins from pituitaries of OVX rats is similar to the response obtained from pituitaries of diestrous II rats.

In spite of the difficulties of interpreting results obtained through the use of pharmacological agents i.e. their lack of specificity and potential toxicity effects, the results of this study are consistent with a role for cAMP as a mediator of GnRH-stimulated gonadotropin secretion. The demonstration that 1) GnRH stimulates cAMP production in pituitaries from OVX rats; 2) Flu inhibits the GnRH-stimulated increases in cAMP production and 3) the replacement of cAMP results in the selective restoration of Flu-induced inhibition of GnRH-stimulated gonadotropin secretion, provide persuasive evidence for a pivotal role for cAMP.

The fact that DBcAMP was unable to stimulate gonadotropin secretion by itself, but was able to stimulate LH and FSH secretion in the presence of GnRH and Flu suggests an indirect role for cAMP in GnRH-stimulated gonadotropin secretion i.e. the nucleotide is dependent on other intracellular mediators for the manifestation of gonadotropin secretion. Furthermore, the DBcAMP-restored response was sensitive to cycloheximide, suggesting that the nucleotide is associated with protein synthesis-dependent mechanisms. Taken together, these observations suggest cAMP probably stimulates the de novo synthesis of proteins which are intimately associated with signal transduction and/or secretory mechanisms. Other intracellular mediators, leukotrienes, Ca^{++}, PKC may then regulate the functions of these proteins, resulting in the secretion of LH and FSH. It should be noted that the protein synthesis-dependent effects of cAMP on the potentiation of GnRH-stimulated LH and FSH secretion reported by several investigators (Cronin et al., 1984; Turgeon and Waring, 1986[a] and DeKoning et al., 1981) are consistent with this concept.

The temporal characteristics and protein synthesis-dependency of the gonadotropin secretion that appears to be indirectly mediated by cAMP suggest that this is the same component that demonstrates synergy between ionomycin and PMA. In other words, cAMP plus the mechanisms activated by Ca^{++} mobilization and PMA all interact to produce this component of gonadotropin secretion from pituitaries of OVX rats. If cAMP does, in fact, play an indirect but obligatory role in the synergistic component of LH and

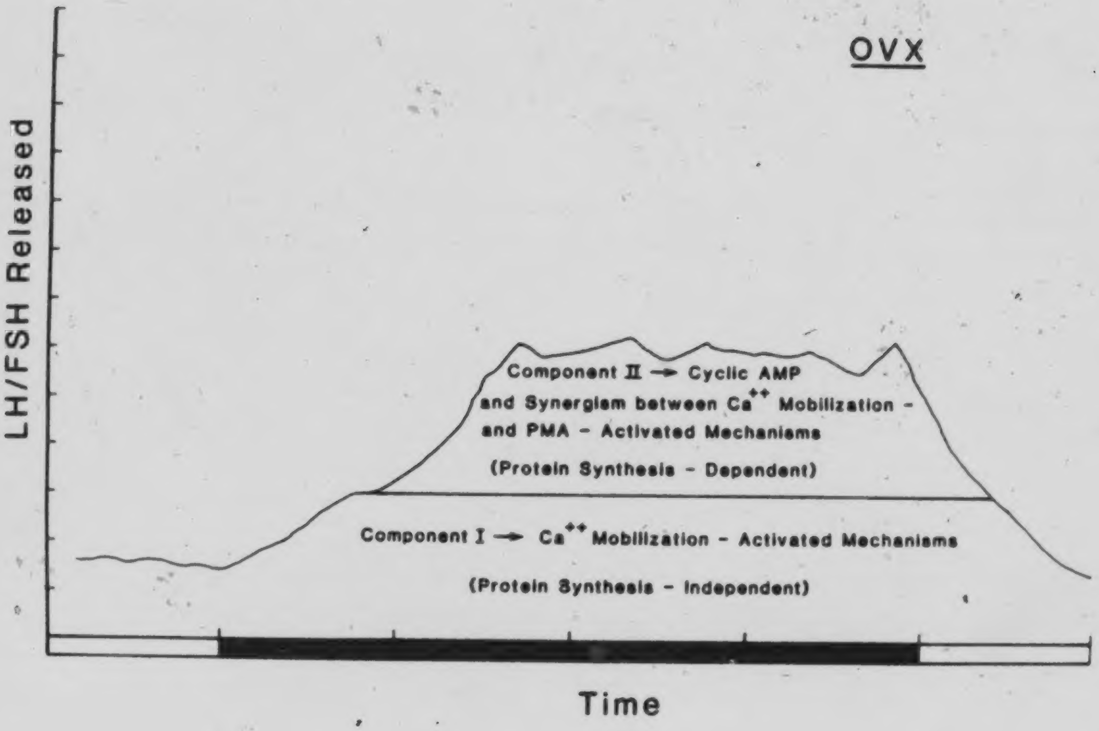
FSH secretion, then either ionomycin and/or PMA would be expected to increase cAMP production in gonadotropes. As discussed previously, experiments are currently being performed to determine if these secretagogues stimulate cAMP production in pituitaries from female rats.

The GnRH-stimulated, protein synthesis-dependent component from pituitaries of OVX rats is attenuated when compared to diestrous II animals (cf. Figs. 11[a,b] and 25[a,b]) (Baldwin et al., 1983). In the previous section, it was suggested that the attenuation could be due (at least in part) to decreased activity of PMA-activated mechanisms. However, estradiol was shown to increase cAMP production (Tang et al., 1982), cAMP-dependent protein kinase (PKA) activity and cAMP binding in pituitary cells of females (Tang and Tang, 1983). Consequently, ovariectomy and the resultant decrease in circulating estradiol concentrations could conceivably result in decreased cAMP production, decreased cAMP binding and decreased PKA activity. As a result, the reduced activity of the adenylyl cyclase-cAMP system could also contribute to the attenuation of secretory responses from pituitaries of OVX rats.

Exogenous administration of DBcAMP potentiated the acute, protein synthesis-independent secretion of LH obtained in the presence of GnRH and Flu. Moreover, the DBcAMP-potentiating effect on the acute response was also protein synthesis-independent. The demonstration that adenosine nucleotide analogs can interact with GnRH receptors (Smith et al., 1982), makes it tempting to speculate that the potentiation of acute GnRH-stimulated gonadotropin secretion might be due to synergistic interactions of the adenosine nucleotides and GnRH at the receptor level.

In conclusion, the results suggest an obligatory but indirect role of cAMP in mediating a phase II component of gonadotropin secretion from pituitaries of OVX rats. The dependency of the cAMP effects on cycloheximide suggests that the nucleotide exerts its effects by stimulating de novo protein synthesis. It is also apparent that the cyclic nucleotide does not mediate the acute, protein synthesis-independent release of gonadotropins. Combining these results with those obtained using ionomycin and PMA, it appears that there is a small, cAMP- and protein synthesis-independent component of gonadotropin secretion which is mediated by Ca^{++} mobilization-activated mechanisms. In contrast, the component of release mediated through synergy of ionomycin and PMA is coupled to cAMP in a protein synthesis-dependent manner (Fig. 27).

FIG. 27. Summary of the secretory profile of LH/FSH from pituitaries of OVX rats indicating the various components and the branches of the Ca^{++} and cAMP messenger systems which mediate them.


OVX
LH/FSH Released
Component II → Cyclic AMP
and Synergism between Ca^{++} Mobilization -
and PMA - Activated Mechanisms
(Protein Synthesis - Dependent)
Component I → Ca^{++} Mobilization - Activated Mechanisms
(Protein Synthesis - Independent)
Time

SUMMARY

The realization that GnRH-stimulated gonadotropin secretion is complex, coupled with the large number of implicated intracellular mediators of GnRH, suggested that gonadotropin secretory profiles might actually be a composite of processes activated by different second messengers. This formed the basis for the present study designed to delineate the roles/ interactions of the branches of the Ca^{++} messenger system as mediators of the biphasic secretion of LH and FSH from pituitaries of diestrous II rats. Additional studies were also undertaken to investigate potential changes in the roles/ interactions of the Ca^{++} and cAMP systems as mediators of gonadotropin secretion following ovariectomy. The objectives were achieved through the use of an in vitro anterior pituitary tissue perifusion system which duplicates in vivo gonadotropin secretory profiles.

Both GnRH and the simultaneous administration of ionomycin + PMA stimulated a biphasic secretion of LH and FSH from pituitaries of diestrous II rats. Furthermore, this composite secretory response was dissociated into i) an initial, small, protein synthesis-independent component (phase I), which appears to be mediated by Ca^{++} mobilization-activated mechanisms ii) a delayed (approximately 60 min), relatively small, protein synthesis-dependent component (phase IIa), mediated by PMA-activated mechanisms e.g. PKC and iii) a delayed (approximately 60 min), large, protein synthesis-dependent component (phase IIb) mediated by synergistic interactions between Ca^{++} mobilization- and PMA-activated mechanisms (Fig. 26).

Ovariectomy resulted in an attenuation of the second phase of gonadotropin secretion. This attenuation appears to be due (at least in part) to the absence of the delayed, protein synthesis-dependent phase II secretion that was directly mediated by PMA-activated mechanisms. The secretory responses obtained from pituitaries of OVX rats included (i) an initial, small, protein synthesis- and cAMP-independent component mediated by the mechanisms activated by Ca^{++} mobilization and (ii) a larger, protein synthesis-dependent component mediated by synergistic interactions between Ca^{++} mobilization- and PMA-

activated mechanisms. The latter component is also mediated by cAMP, which plays an obligatory, pivotal but indirect role which is associated with *de novo* protein synthesis.

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APPENDIX 1

Composition of Kreb's Improved Ringer I Bicarbonate (KIRB) Medium

80 parts of 0.154 M sodium chloride

4 parts of 0.154 M potassium chloride

3 parts of 0.11 M calcium chloride

1 part of 0.154 M potassium dihydrogen phosphate

1 part of 0.154 M magnesium sulphate

21 parts of 0.31 M sodium bicarbonate

4 parts of 0.16 M sodium pyruvate

7 parts of 0.05 M sodium fumarate

4 parts of 0.16 M sodium glutamate

5 parts of 0.30 M α D (+) glucose

2.68 parts of MEM amino acid stock (as supplied by Flow Laboratories, cf. Materials and Methods)

1.34 parts of MEM vitamins stock (as supplied by Flow laboratories, cf. Materials and Methods).

APPENDIX 2

Data and log-logit plots for representative radioimmunoassays for LH and FSH.

RADIOIMMUNOASSAY FOR LH

PERCENT OF TOTAL BOUND = 0.29

CONCS.	COUNTS	COUNTS	COUNTS	COUNTS	COUNTS	MEAN COUNTS
TOTALS	9655	9549	9616	9418	9339	9515
NSB	79	97	118	103	94	98
B0	2840	2836	2836	2804	2784	2820

CONCS.	COUNTS	COUNTS	COUNTS	MEAN COUNTS	PERCENT BOUND	LOGIT
0.0625 ng	2478	2407	2424	2436	0.859	1.81
0.1250 ng	2178	2201	2193	2191	0.769	1.20
0.2500 ng	1813	1772	1830	1805	0.627	0.52
0.5000 ng	1405	1359	1331	1365	0.465	-0.14
1.0000 ng	906	904	881	897	0.293	-0.88
2.0000 ng	565	579	593	579	0.177	-1.54

UNWEIGHTED REGRESSION ANALYSIS

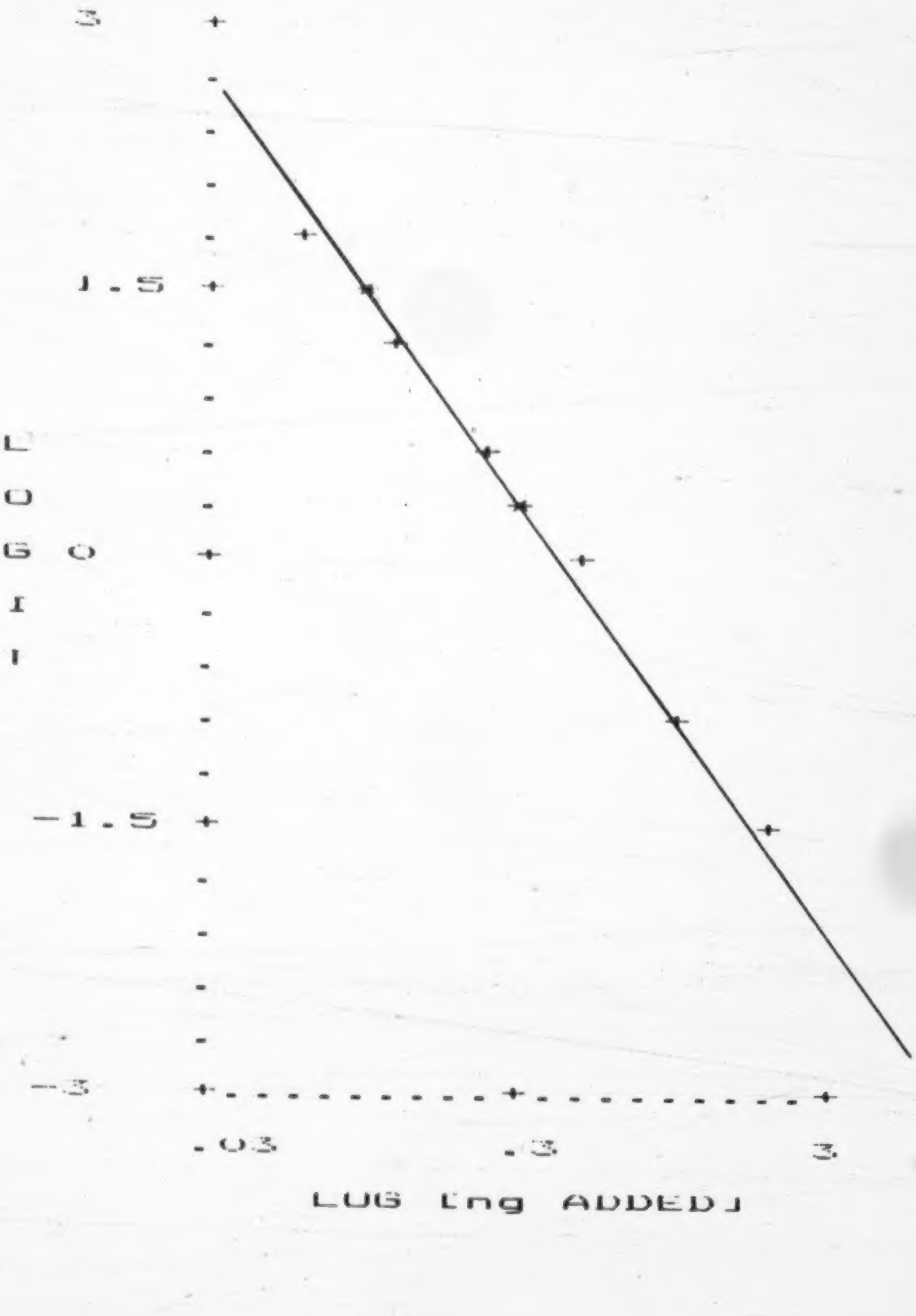
CORRELATION COEFFICIENT = 0.9993
Y INTERCEPT = -.8507
SLOPE = -2.2429

WEIGHTED REGRESSION ANALYSIS

Y INTERCEPT = -.8549
SLOPE = -2.2763

10 PERCENT (ED 10) = 3.89
50 PERCENT (ED 50) = 0.42
90 PERCENT (ED 90) = 0.05

LH RIA



RADIOIMMUNOASSAY FOR FSH

PERCENT OF TOTAL BOUND = 0.21

CONCS.	COUNTS	COUNTS	COUNTS	COUNTS	COUNTS	MEAN COUNTS
TOTALS	10377	10596	10482	10530	10530	10503
NSB	80	128	141	120	95	113
BO	2229	2244	2303	2201	2316	2259

CONCS.	COUNTS	COUNTS	COUNTS	MEAN COUNTS	PERCENT BOUND	LOGIT
0.3125 ng	2106	2017	1979	2034	0.895	2.15
0.625 ng	1863	1863	1978	1901	0.834	1.61
1.25 ng	1614	1616	1641	1624	0.704	0.87
2.5 ng	1219	1208	1245	1224	0.518	0.07
5.0 ng	763	826	822	804	0.322	-0.74
10.0 ng	503	483	498	495	0.178	-1.53

UNWEIGHTED REGRESSION ANALYSIS

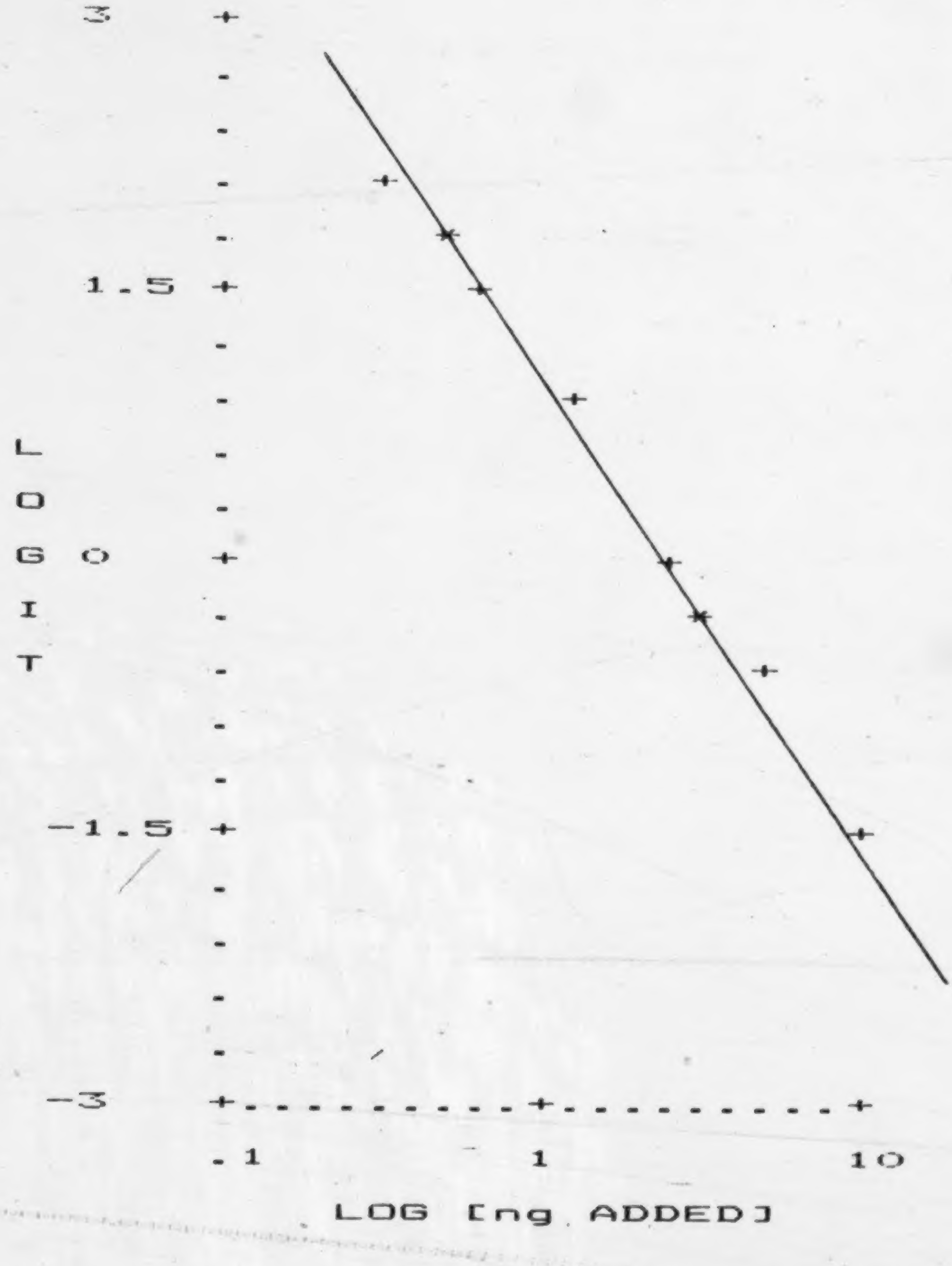
CORRELATION COEFFICIENT = 0.9962
Y INTERCEPT = 1.0197
SLOPE = -2.4910

WEIGHTED REGRESSION ANALYSIS

Y INTERCEPT = 1.0877
SLOPE = -2.6079

10 PERCENT (ED 10) = 18.18
50 PERCENT (ED 50) = 2.61
90 PERCENT (ED 90) = 0.38

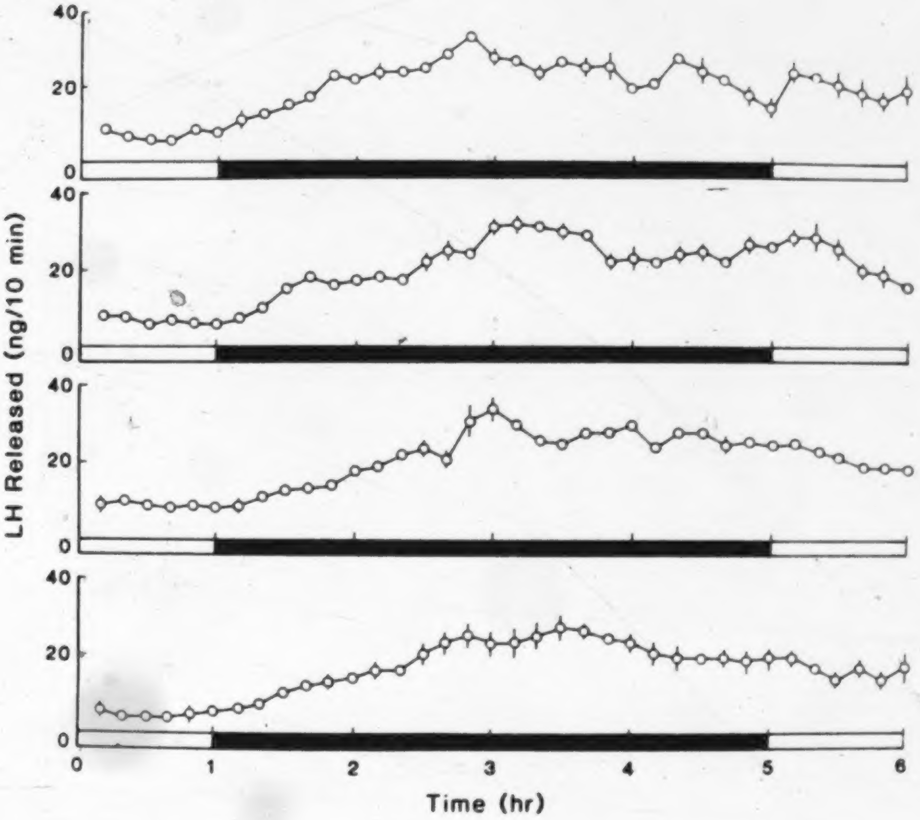
FSH RIA



APPENDIX 3

Reproducibility of the secretory profiles obtained from pituitaries of diestrous II-rats in response to continuous infusions (4 h) of 10 μM ionomycin in KIRB medium. Dates when the experiments were done, from top to bottom:

January 8. 1990.
May 25, 1989.
April 20, 1989.
February 12, 1988.


LH Released (ng/10 min)
40
20
0
0
1
2
3
4
5
6
Time (hr)